In vitro response to ACTH of the interrenal tissue of rainbow trout (Oncorhynchus mykiss) exposed to cadmium

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Abstract

Plasma cortisol levels and responsiveness of the interrenal tissue to ACTH were determined in rainbow trout (Oncorhynchus mykiss) subjected to acute and subchronic exposures of cadmium (Cd) in the water. Plasma cortisol levels were significantly increased after 2 days of exposure to 1 \( \mu \text{g} \text{Cd} \text{dl}^{-1} \). They decreased to basal levels after 7 and 14 days of exposure. They significantly increased again after 30 days of exposure. The responsiveness of the interrenal tissue to ACTH was evaluated in vitro by monitoring the secretion of cortisol after stimulation of the head kidneys with \( 10^{-7} \text{ M} \) of ACTH for 10 min in perifusion. The responsiveness to ACTH of the interrenal tissue of fish exposed to 1 \( \mu \text{g} \text{Cd} \text{dl}^{-1} \) for 2, 7 and 14 days and 5 \( \mu \text{g} \text{Cd} \text{dl}^{-1} \) for 7 days was not significantly different from controls. On the other hand, the interrenal tissue of fish exposed to 1 \( \mu \text{g} \text{Cd} \text{dl}^{-1} \) for 30 days secreted significantly larger amounts of cortisol in response to ACTH compared to controls. The significance of these findings to the interrenal dysfunction previously diagnosed in fish from lakes contaminated by heavy metals is discussed. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Fish; Cortisol; ACTH; In vitro test; Cadmium

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1. Introduction

Cadmium (Cd) is a widely distributed heavy metal, toxic to terrestrial and aquatic organisms (Wright and Welbourn, 1994). In fish, uptake of Cd occurs across the gastrointestinal tract and gills where it involves Ca^{2+} channels (Verbost et al., 1989; Pratap and Wendelaar Bonga, 1993). An exposure of fish to Cd can lead to: (i) a disturbance of ion and water balance; (ii) anemia; (iii) impaired synthesis of sex steroids; and (iv) vertebral deformities (Sangalang and O’Halloran, 1972; Bengtsson et al., 1975; Giles, 1984; Haux and Larsson, 1984; Sjöbeck et al., 1984).

Numerous studies reported an elevation of plasma cortisol levels in fish exposed to Cd (James and Wigham, 1986; Pratap and Wendelaar Bonga, 1990; Fu et al., 1990; Gill et al., 1993; Hontela et al., 1996; Ricard et al., 1997). This increase is a characteristic response of fish to toxicants and to a variety of other stressors such as handling, confinement and variations of pH and dissolved oxygen (Donaldson, 1981; Hontela 1997). The activation of cortisol secretion is part of a general adaptation response to stressors which facilitates—through the osmoregulatory (Flick and Perry, 1989; Laurent and Perry, 1990) and gluconeogenic (Leach and Taylor, 1982) actions of cortisol—maintenance of homeostasis in the presence of an external disturbance. When the doses of the toxicant are acutely lethal, the rise in plasma cortisol is rapid and pronounced. It is maintained until death ensues (Donaldson, 1981; Brown, 1993). At longer laboratory exposures to sublethal doses, the elevation of plasma cortisol is however frequently followed by a decline to basal levels and a secondary rise of plasma cortisol is sometimes observed (Donaldson, 1981; Brown, 1993; Hontela, 1997). In fish chronically exposed in the field to organic pollutants and/or heavy metals, plasma cortisol levels are low and the capacity of the interrenal tissue to respond to adrenocorticotropic hormone (ACTH)—the endogenous stimulus for cortisol secretion by the interrenal tissue—is significantly reduced (Lockhart et al., 1972; Brodeur et al., 1997a,b; Girard et al., 1997). These results suggest that a life-long exposure to sublethal levels of toxicants may lead to an impairment of the normal function of the interrenal tissue.

The mechanisms underlying the interrenal dysfunction in fish from polluted sites have not been elucidated so far. Furthermore, it is not known if functional modifications occur within the interrenal tissue when plasma cortisol levels are increased or decreased during laboratory exposures to Cd. Metals may exert direct toxic effects in the interrenal cells since toxicity of: Cd; mercury; and copper have been demonstrated in the steroidogenic cells of the adrenal gland of mammals, the homologous of fish interrenal tissue (Veltman and Maines, 1986a,b; Mgbonyebi et al., 1994). A chronic elevation of plasma cortisol levels and the synthetic activity of the interrenal cells may however also lead to a functional exhaustion of the steroidogenic capacity of the interrenal cells. To elucidate the mechanisms through which Cd modifies cortisol secretion and to understand how wild fish chronically exposed to heavy metals develop an impairment of the interrenal tissue, the effects of acute (days and weeks) and subchronic (1 month) exposures to sublethal levels of Cd on the responsiveness of trout interrenal tissue to ACTH were assessed in vitro in this laboratory study.
2. Methods

2.1. Fish and maintenance regimens

Immature male and female rainbow trout, Oncorhynchus mykiss (mean body weight ± S.D.; 53 ± 21.4 g) were obtained from a hatchery and maintained for 2 weeks in flow-through (455 l) holding tanks (water hardness 135 mg l\(^{-1}\) CaCO\(_3\), temperature 10–12°C, photoperiod 12L:12D with onset of the light phase at 07:30 h, dissolved oxygen at saturation, pH 7.2). The water flow rate insured 99% replacement every 2 h, about 4.3 l\(^{-1}\) day\(^{-1}\). Fish were fed commercial trout chow daily between 16:00 and 18:00 h at a rate of 1% of the average body weight. Following the 2 weeks, fish were transferred at random into 70-l flow-through experimental tanks (12–14 fish/tank) and were acclimated for 2 weeks before starting the exposure to Cd.

2.2. Experimental treatments

In a first series of acute exposures, fish were exposed through water to 0 and 1 \(\mu\)g Cd l\(^{-1}\) as CdCl\(_2\) for 2, 7 and 14 days or to 0, 1 and 5 \(\mu\)g Cd l\(^{-1}\) for 7 days (1 tank/treatment). A subchronic exposure to 0 and 1 \(\mu\)g Cd l\(^{-1}\) for 30 days was conducted in a second series of experiments. The water Cd concentrations were maintained at a constant level with Mariotte bottles (Hontela et al., 1996). The Cd concentrations were measured by atomic absorption by the procedure described in Manca et al. (1992), modified for water samples acidified with ultrapure nitric acid.

2.3. Sampling

Fish were always sampled between 09:00 and 11:00 h to minimize the effects of the diel cycles of cortisol secretion. At the end of the exposure period, all the fish in an experimental tank were removed at once. They were held for 5 s in the net to standardize the handling procedure and they were anesthetized by immersion in a solution of tricaine methanesulfonate (MS-222, 0.1 g l\(^{-1}\) of water). Fish were weighed and a blood sample was taken from the caudal vasculature with a heparinized syringe. The plasma was separated by centrifugation (5 min, 13000 rpm), immediately frozen on dry ice and kept frozen at −80°C until cortisol was measured.

2.4. Responsiveness of the interrenal tissue to ACTH—\textit{in vitro} test

Immediately after fish were blood sampled. The head kidneys containing the interrenal tissue were dissected to evaluate \textit{in vitro} using perifusion, the functional integrity of the interrenal tissue. The perifusion procedure has been described in detail elsewhere (Brodeur et al., 1997a). Head kidneys are placed in an incubation chamber connected to a peristaltic pump continuously supplying the tissues with fresh incubation medium. After perifusion for 110 min to reach the basal rate of
cortisol secretion, the interrenal tissue is stimulated by adding $10^{-7}$ mol l$^{-1}$ of porcine ACTH$^{1-39}$ to the incubation medium for 10 min. The response of the interrenal tissue is monitored by collecting the medium continuously evacuated from the chamber. This is done in 10 min fractions for 120 min and measuring the cortisol concentrations in the fractions by radioimmunoassay. The sum of cortisol measured in all the fractions is used to compare the response to ACTH of fish from different groups. The head kidneys from two animals were pooled in each perfusion chamber.

2.5. Radioimmunoassay of cortisol

Concentrations of cortisol in plasma and in the incubation medium collected from perfusion were determined with a radioimmunoassay kit (ICN Biomedicals No. 07-221102). A standard curve prepared by adding synthetic cortisol (Sigma, St. Louis, MO) to the medium at concentrations used in the kit’s standard curve was used to measure cortisol in samples collected from perfusion. The characteristics of the assays done with this standard curve were described previously (Brodeur et al., 1997a).

2.6. Statistical analyses

Differences between groups were tested by either $t$-test or one-way and two-way ANOVA. A Student–Newman–Keuls (SNK) test was used to execute multiple comparisons when the ANOVA detected a significant difference. The data were log-transformed to attain normality and homoscedasticity when necessary.

3. Results

3.1. Cadmium concentrations in water

Exposure was characterized by measuring Cd levels in water of the treatment tanks during the experiments. The average concentrations of Cd (mean ± S.E.) measured throughout the experiments for the groups treated with 0, 1 and 5 μg Cd l$^{-1}$ were, respectively $0.22 ± 0.6$ ($n = 9$), $1.0 ± 0.4$ ($n = 8$) and $5.9 ± 1.2$ ($n = 2$) μg Cd l$^{-1}$.

3.2. Exposure to 1 and 5 μg Cd l$^{-1}$

Plasma cortisol levels of fish exposed to 1 μg Cd l$^{-1}$ for 2 days were significantly higher ($P < 0.05$) than the levels measured in fish from the control group (Fig. 1A). After 7 days, plasma cortisol levels of fish exposed to 1 μg Cd l$^{-1}$ were decreased ($P < 0.05$) compared to the levels measured after 2 days of exposure. There were no significant differences between the control group and groups exposed to 1 or to 5...
Fish exposed to 1 μg Cd l⁻¹ for 14 days had lower plasma cortisol than the controls which had slightly higher levels ($P < 0.05$) compared to controls on days 2 and 7.

The interrenal tissues of controls and fish exposed to 1 or 5 μg Cd l⁻¹ for 2, 7 or 14 days exhibited a similar responsiveness to ACTH since the amounts of cortisol they secreted in response to 10 min of perifusion with $10^{-7}$ M of ACTH were not significantly different (Fig. 1B). A trend for a decrease in cortisol secretion related to the dose of Cd was however apparent on day 7.

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Fig. 1. Exposure of juvenile rainbow trout to 0, 1 and 5 μg Cd l⁻¹ for 2, 7 and 14 days. (A) Plasma cortisol levels (mean ± S.E.). (B) Cortisol (mean ± S.E.) secreted by the head kidneys after stimulation with $10^{-7}$ M of ACTH during 10 min in perifusion. Numbers of fish sampled are indicated on top of the error bars. * Significantly different from the control group sampled on the same day and # Significantly different from the two other exposure durations (ANOVA II + SNK test, $P < 0.05$).
Fig. 2. Exposure of juvenile rainbow trout to 0 and 1 μg Cd l⁻¹ for 30 days. (A) Plasma cortisol levels (mean ± S.E.). (B) Cortisol (mean ± S.E.) secreted by the head kidneys after stimulation with 10⁻⁷ M of ACTH during 10 min in perifusion. Numbers of fish sampled are indicated on top of the error bars. * Significantly different from the control group (t-test, P < 0.05).

3.3. Exposure to 1 μg Cd l⁻¹ for 30 days

Trout exposed to 1 μg Cd l⁻¹ for 30 days had significantly higher levels of plasma cortisol compared to fish from the control group (Fig. 2A). The interrenal tissue of fish exposed to Cd (1 μg l⁻¹) for 30 days was also more responsive to ACTH than the interrenal tissue of fish from the control group since it secreted a significantly higher amount of cortisol in response to ACTH (Fig. 2B). The time-course of cortisol secretion by the perifused interrenal tissue of control and treated fish was however similar (Fig. 3).
4. Discussion

The concentrations of Cd used in the present study (1 and 5 μg l⁻¹) are sublethal and represent less than 0.5% of 4 mg Cd l⁻¹, the estimated value of the 48 h LC₅₀ of rainbow trout maintained in water at 135 mg CaCO₃ l⁻¹. The value of 4 mg Cd l⁻¹ was derived from 48 h LC₅₀s reported for rainbow trout by Pascoe et al. (1986), specifically 2.9 mg Cd l⁻¹ in water of 70 mg CaCO₃ l⁻¹ and 5.7 mg Cd l⁻¹ in water of 279 mg CaCO₃ l⁻¹. No mortality was recorded during the exposures to Cd. Unpolluted lakes have water concentrations of Cd generally lower than 0.1 μg l⁻¹ (Tessier et al., 1993; Wright and Welbourn, 1994). Concentrations of Cd of 0.4–0.5 μg l⁻¹ are frequently observed in areas where mining and smelting activities occur. Concentrations of 5 μg l⁻¹ and more have been reported (Tessier et al., 1993). Fish from polluted environments are also exposed to Cd through their food, unlike fish exposed in the laboratory. The concentrations of Cd used in the present study (1 and 5 μg Cd l⁻¹) can thus be considered as representative of environmental exposures.

The acute (2 days) exposure to 1 μg Cd l⁻¹ significantly elevated plasma cortisol levels in the rainbow trout. This increase in response to Cd is a phenomenon that has been previously observed in several fish species (James and Wigham, 1986; Pratap and Wendelaar Bonga, 1990; Fu et al., 1990; Gill et al., 1993; Hontela et al., 1996; Ricard et al., 1997) but not in salmon, Oncorhynchus kisutch (Schreck and

![Fig. 3. Cortisol secreted (mean) by head kidneys of juvenile rainbow trout from control group (n = 6) and a group exposed to 1 μg Cd l⁻¹ for 30 days (n = 6) during 10-min intervals after stimulation with ACTH in perifusion. The 10-min interval during which ACTH (10⁻⁷ M) was administered is indicated by a bar.](image-url)
The stimulation of cortisol secretion may result from altered osmo-ionic balance since exposure to Cd usually leads to decreased plasma levels of Ca\(^{2+}\) and, to a lesser extent, Na\(^+\) (Haux and Larsson, 1984; Giles 1984; Fu et al., 1990; Ricard et al., 1997). Inhibition of Ca\(^{2+}\) uptake and Na\(^+\)/K\(^{+}\)-ATPase in the gills has been demonstrated in fish exposed to Cd (Verbost et al., 1989; Pratap and Wendelaar Bonga, 1993). Changes in osmotic pressure and ion concentrations stimulate cortisol secretion (Decourt and La hlou, 1986). In turn, cortisol induces proliferation of chloride cells thus increasing influx of Na\(^+\), K\(^{+}\) and Ca\(^{2+}\) (Flick and Perry, 1989; Laurent and Perry, 1990). These actions of cortisol favor the osmo-ionic homeostasis and together with the gluconeogenic action of cortisol, may help the fish to cope with the toxic effects of Cd during acute exposures. An induction of metallothionein synthesis by cortisol, as has been observed in mammals (Hidalgo et al., 1988), would facilitate the process of detoxification.

Despite the continuous presence of Cd in the water, plasma cortisol returned to basal levels after 7 to 14 days of exposure to 1 or 5 µg Cd \(1^{-1}\) in the present study. The decrease of plasma cortisol levels did not result from an impairment of the functional integrity of the interrenal tissue since the in vitro responsiveness to ACTH of the interrenal tissue of fish exposed to Cd was similar to the one of control fish. Thus, the toxic effects demonstrated for Cd and other heavy metals in the adrenal gland of mammals (Veltman and Maines, 1986a,b; Mgbonyebi et al., 1994) were not observed in the interrenal tissue of trout at the doses of Cd and length of exposure used in the present study. The return of plasma cortisol to basal levels could be mediated by the negative feedback effect (Donaldson, 1981; Bradford et al., 1992) exerted on the hypothalamo-pituitary-interrenal axis (HPI axis) by the elevated plasma cortisol levels observed at the beginning of the exposure. The in vitro responsiveness of the interrenal tissue to ACTH was not significantly reduced after 14 days of exposure to Cd in the present study. The feedback effect most likely reduced the secretion of ACTH by the pituitary gland rather than modifying the sensitivity of the interrenal tissue to ACTH. In a study with tilapia (\textit{Oreochromis mossambicus}), Fu et al. (1990) concluded that the return of plasma cortisol to basal levels illustrated that fish have developed tolerance to Cd. It coincided with a return of plasma Ca\(^{2+}\), Na\(^+\) and glucose to near normal levels and a rise in the metallothionein-like protein content of gill, kidney and liver. These parameters were not measured in the present study. However the results show that these compensatory mechanisms may not be efficient enough to protect the fish during extended exposures to Cd since plasma cortisol levels increased again after 30 days of exposure to 1 µg Cd \(1^{-1}\).

The elevation of plasma cortisol levels observed after 30 days of exposure to Cd was associated with an increased in vitro responsiveness of the interrenal tissue to ACTH. The subchronic exposure of fish to Cd thus induced functional modifications within the interrenal tissue. This was in contrast to acute exposures which had no major effects on the responsiveness of the interrenal tissue to ACTH. The mechanisms through which changes in the response to ACTH are mediated have not yet been elucidated in fish. Studies with mammals demonstrated that a chronic stimulation of the adrenal gland with ACTH elevates the concentrations of cy-
tochrome P-450. This cytochrome is responsible for the synthesis of glucocorticoids—by enhancing the transcription of their coding genes (Simpson and Waterman, 1988). It is not presently known whether the secretion of ACTH is stimulated in the trout after 30 days of exposure to Cd and the synthesis of steroidogenic cytochrome P-450s is induced, as has been observed in mammals.

The present study provided evidence that a 30 days exposure to 1 μg Cd l⁻¹ does not functionally impair the interrenal tissue of trout. The absence of toxic effects after 30 days of exposure is in agreement with the previous observation that the impairment of cortisol secretion detected at field sites contaminated by metals is only evident in fish 4+ and older (Brodeur et al., 1997b). The possibility remains that a laboratory exposure to Cd longer than 30 days will cause toxic effects in the interrenal tissue and studies in progress will test this hypothesis. The results obtained in the present study also demonstrated that rainbow trout does not acclimate to water levels of 1 μg Cd l⁻¹ and that cortisol secretion is increased after 30 days of exposure. Sustained stimulation of cortisol secretion and the high metabolic activity of the interrenal tissue may eventually lead to a functional interrenal exhaustion which may not be toxicant-specific. The diagnosis of the interrenal impairment in fish subjected to life-long field exposures to heavy metals (Lockhart et al. 1972; Brodeur et al., 1997a,b; Girard et al., 1997) and also in fish and amphibians tested at sites contaminated by other types of contaminants (McMaster et al., 1994; Hontela et al., 1997; Gendron et al., 1997) supports this hypothesis.

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