Recombinant interleukin-1 β activates the hypothalamic–pituitary–interrenal axis in rainbow trout, *Oncorhynchus mykiss*

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Abstract

The present study provides the first direct evidence that implicates fish cytokines as the effector molecules by which the immune system signals the neuroendocrine system and activates the hypothalamic–pituitary–interrenal stress axis. I.p. injections of trout recombinant interleukin-1 β (rIL-1 β) or *E. coli* lipopolysaccharide (LPS), at concentrations known to induce immune/inflammatory responses *in vivo* (0.1–0.6 nmol/kg and 1.3 mg/kg respectively), significantly elevated plasma cortisol levels in a dose- and/or time-dependent manner. However, in contrast to general stress responses in fish, under the conditions employed in this study, no specific treatment effects on plasma glucose levels could be demonstrated. The trout IL-1 β peptides (P1 and P3), which are homologous to

receptor-binding sequences of human IL-1 β , failed to influence the prevailing cortisol concentration even though an equivalent dose has been found to have immunostimulatory properties *in vivo*. Blockade of endogenous ACTH release by administration of the synthetic glucocorticoid dexamethasone prevented the rIL-1 β /LPS-mediated elevation of plasma cortisol, suggesting that IL-1 β and LPS modulate cortisol secretion via effects at the level of the hypothalamic–pituitary axis. These data indicate that, with respect to IL-1 β , cytokine signalling between the immune and neuroendocrine systems in mammals appears to be conserved in lower vertebrates.

Journal of Endocrinology (2002) **175**, 261–267

Introduction

Proinflammatory cytokines in mammals, particularly interleukin (IL)-1 β , have been identified as the effector molecules by which the immune system signals the hypothalamic–pituitary–adrenal (HPA) axis, primarily with respect to the stimulated release of hypothalamic corticotrophin-releasing hormone (CRH) (Shintani *et al.* 1995, Turnbull & Rivier 1999). Indeed, the systemic and central nervous system (CNS) generation of IL-1 β in response to endotoxin treatment and to physical or psychological stressors is known to contribute towards the associated HPA secretory activity (Shintani *et al.* 1995, Turnbull & Rivier 1999). However, the efficacy of cytokine action within the HPA axis depends on the prevailing glucocorticoid concentration (Buckingham *et al.* 1994, 1996), since the HPA axis is subjected to feedback control by glucocorticoids at the hypothalamic–pituitary level (Sapolsky *et al.* 2000). In addition to the effects on the HPA axis, there is also evidence that peripherally administered IL-1 β may influence glucose metabolism, causing a marked and prolonged hypoglycaemic response (Rey & Besedovsky 1987).

In fish, research into the cross-talk between the immune and neuroendocrine systems has focused on the modulation of immune and inflammatory processes by hormones and the expression of their receptors in fish leucocytes, particularly with respect to the interrenal glucocorticoid, cortisol (Wendelaar-Bonga 1997, Weyts *et al.* 1999). However, in contrast to understanding the effects of fish hormones, progress in examining the impact of immune-derived signals on the neuroendocrine system has been greatly hampered by the absence of cloned fish cytokine genes and the production of their recombinant proteins, although the cytokine-inducing mitogen lipopolysaccharide (LPS) is known to stimulate hypothalamic–pituitary–interrenal (HPI) secretory activity in fish (Balm 1997).

Recently, the IL-1 β gene sequence in both rainbow trout (Zou *et al.* 1999a,b) and carp (Fujiki *et al.* 2000) was elucidated, thus enabling the production of functional fish IL-1 β recombinant proteins. Since then, the gene has been cloned in a number of other fish including the first sequence from a cartilaginous species (Secombes *et al.* 2001, Bird *et al.* 2002). *In vitro* and *in vivo* studies with trout recombinant IL-1 β (rIL-1 β), to date, have

demonstrated the potency of this molecule in inducing a range of immune genes (e.g. IL- β , cyclooxygenase-2 and MHC class II β chain) and stimulating phagocytosis, leucocyte bactericidal activity and leucocyte migration (Hong *et al.* 2001, Peddie *et al.* 2001, Hong 2002). Furthermore, short IL-1 β peptides (P1 and P3) have been constructed to correspond to areas within the trout rIL-1 β molecule that are predicted to interact with the signal-transducing binding site of the IL-1 receptor (Secombes *et al.* 1998). In a similar manner to the whole rIL-1 β molecule, the peptide P3 has been found to have profound immunostimulatory properties both *in vitro* and *in vivo*, with evidence of synergy occurring between P1 and P3 (Peddie *et al.* 2001, 2002, Hong 2002, Peddie 2002).

In view of the lack of knowledge regarding the role of cytokines in the neuroendocrine stress response of fish, the present work was undertaken to investigate the *in vivo* activity of administered trout rIL-1 β , IL-1 β peptides (P1 and P3) and LPS by measuring plasma cortisol and glucose levels in fish with both intact and dexamethasone (DEX)-blocked HPI axes.

Materials and Methods

Fish and reagents

A mixed-sex population of sexually immature rainbow trout *Oncorhynchus mykiss* (100–300 g), reared at the Centre for Ecology and Hydrology, Windermere, Cumbria, UK, was maintained in 1000 l outdoor tanks supplied with a constant flow of lake water (25 l/min) at mean temperatures of 13.9 ± 0.2 °C (mean $\pm$ S.E.; initial time-course experiment) and 14.8 ± 0.1 °C (mean $\pm$ S.E.; DEX-block study). The fish were fed three times weekly (Trouw Standard Expanded 40, Trouw Aquaculture, Wincham, UK) at the manufacturers' recommended rate. Feeding was discontinued during the experiments. The synthetic IL-1 β peptides P1 (YVTPVPIETEAR) and P3 (YRRNTGVDIS), corresponding to a molecular mass of 1.37 kDa (amino acids 146–157) and 1.18 kDa (amino acids 207–216) respectively, were designed and produced as described previously (Peddie *et al.* 2001). The batches of peptides used have been shown to influence immune gene expression and leucocyte function (Hong 2002, Peddie 2002) at a concentration equivalent to the dose used in this study (see below). [1,2,6,7- 3 H]cortisol (2.22 TBq/mmol) was purchased from Amersham Pharmacia Biotech UK, and cortisol antibody (IgG-F-2) was purchased from Campro Scientific (Veenendaal, The Netherlands). All other reagents were obtained from Sigma unless otherwise stated.

Production of trout rIL-1 β

Trout rIL-1 β was purified using a modified protocol from the method described by Hong *et al.* (2001). The six

histidine-tagged cDNA sequence encoding the predicted mature IL-1 β peptide was cloned into the vector pQE30 (Invitrogen) and transformed into *E. coli* cells (JM109; Promega). Clones containing the insert were selected by PCR screening and plasmid DNA was sequenced to confirm that there had been no alteration of the coding domain. Cells from a single colony were cultured in 10 ml LB medium containing ampicillin (50 μ g/ml) and shaken (200 r.p.m.) overnight at 37 °C followed by culture in 500 ml LB medium for 4 h at 37 °C. Recombinant protein production was induced following the addition of 1 mM isopropyl- β -D-thiogalactoside to the cells followed by a further incubation of 4 h at 37 °C. Cells were harvested and resuspended in 5 ml lysis buffer (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, 20 mM imidazole, pH 8.0), and mixed continually for 60 min at room temperature. The cell lysate was applied to a Ni-nitriloacetic acid column (Qiagen) equilibrated with lysis buffer. Proteins lacking the His tag were eluted using lysis buffer solutions (pH 6.3 and 5.9 successively). The immobilized IL-1 β was refolded by washing repeatedly with buffer containing 50 mM Tris-HCl, 500 mM NaCl, 5 mM MgCl₂, 20% glycerol and 20 mM imidazole, pH 8.0. Bound recombinant protein was eluted using buffer containing 250 mM imidazole.

Protein levels of the flow-through fractions were determined (Bradford 1976) and the size and purity of the eluted protein was confirmed by SDS-PAGE. Eluted fractions exhibited a single protein band with a molecular mass of approximately 20 kDa, in agreement with previous studies (Hong *et al.* 2001, Peddie *et al.* 2001). Using a *Limulus* amebocyte lysate kit (Sigma), the LPS levels detected in rIL-1 β preparations have been found to be consistently less than 16 ng/ml and are, therefore, unlikely to interfere with the bioactivity of the rIL-1 β preparation since far greater concentrations are needed to induce effects in trout leucocytes (Zou *et al.* 2000).

I.p. injection of trout rIL-1 β , IL-1 β peptides and E. coli LPS

Two weeks prior to the experiment, 64 fish were distributed evenly between eight tanks, containing 500 l water. At the start of the study, fish were removed from each tank in turn, anaesthetized (2-phenoxyethanol, 1:2000 v/v), marked on the ventral surface with alcian blue dye using a Panjet needleless injector (Wright Dental Group, Manchester, UK) for subsequent identification, and injected with one of eight treatments before being returned to their holding tank. Each tank, therefore, contained eight fish each receiving one of eight treatments, thereby ensuring that all treatments were replicated across tanks, avoiding the possibility of unaccounted-for tank effects. In order to minimize the disturbance associated with capture of the fish throughout the study, anaesthetic was injected directly into the incoming water supply to each tank. After a period to allow for mixing, the water supply was shut off

and the immobilized fish were rapidly removed from the tank using a dipnet. The eight treatments administered to fish by i.p. injection in a total volume of 400 μ l of vehicle were: (i) trout rIL-1 β (0.1 nmol/kg); (ii) trout rIL-1 β (0.6 nmol/kg); (iii) IL-1 peptide P1 (5.1 μ mol/kg); (iv) IL-1 peptide P3 (5.1 μ mol/kg); (v) LPS (*E. Coli* 0127:B8, 1.3 mg/kg); (vi) IL-1 β peptides P1 and P3 evenly mixed and injected at a final dose of 5.1 μ mol/kg (to test for possible synergistic effects); (vii) recombinant elution buffer (REB, vehicle for rIL-1 β); (viii) PBS (vehicle for LPS and IL-1 β peptides). Blood samples from each fish ($\sim$ 500 μ l) were taken from the cuverian sinus into a heparinized syringe immediately before injection and at 8, 24, 48 and 72 h thereafter. Blood was kept on ice until centrifugation and plasma samples were stored at -20°C prior to cortisol and glucose determination.

DEX blockade

In order to further investigate the interaction of rIL-1 β and LPS with the HPI axis in trout, the cortisol agonist DEX was utilized. DEX is a potent cortisol agonist, suppressing the release of adrenocorticotrophic hormone (ACTH) by the pituitary via negative feedback exerted at the hypothalamic/pituitary level. It possesses the additional advantage of failing to cross-react in the cortisol RIA, therefore allowing the determination of endogenous cortisol levels in DEX-treated fish. Thirty-two fish were divided evenly between four tanks as described above. At 24 h prior to i.p. injection with rIL-1 β (0.6 nmol/kg) or LPS (1.3 mg/kg), four fish in each tank received DEX (1 mg/kg in PBS:ethanol, 3:1; 150 μ l) or vehicle only. Blood samples were collected from the fish at 3, 6 and 8 h after treatment for cortisol and glucose analysis. To confirm that the DEX treatment was effective in blocking the endogenous pituitary/interrenal response to stress, four groups of eight fish from the experimental stock were injected with DEX as described above. At 24 h after the injection, the fish were transferred from their holding tanks, in groups of eight, to four 50 l confinement tanks, a protocol widely used to elicit a stress response in fish (Pottinger & Carrick 1999). After 2 h the fish were anaesthetized and blood samples were collected for cortisol analysis.

Glucose and cortisol determinations

Plasma glucose concentrations were measured using the glucose oxidase (Trinder) method (Sigma). Cortisol levels were determined in plasma by RIA (Pickering *et al.* 1987).

Statistical analysis

Data were analysed by ANOVA (Genstat 5; Lawes Agricultural Trust, Rothamsted Experimental Station, UK) with individual fish, tank, treatment (LPS/peptide or

vehicle), DEX (DEX or vehicle) and time of sample as factors where appropriate. In all cases a plot of residuals against fitted values indicated that mean and variance were co-dependent. Therefore, data were log-transformed prior to analysis. Significant differences between groups were determined using the estimated standard error of the differences between means, derived from the ANOVA.

Results

Blood cortisol levels were significantly elevated at 8 h after injection with rIL-1 β at a dose of 0.6 nmol/kg, relative to sham-injected controls (Fig. 1a; $P<0.02$). Similarly, blood cortisol levels were also markedly increased 8 h after injection with LPS, relative to the PBS-injected control group (Fig. 2a; $P<0.05$). In both cases, the stimulatory effects were no longer apparent at 24 h with plasma cortisol returning to basal levels by 72 h, although preliminary data obtained from LPS/rIL-1 β experiments conducted at a mean water temperature of 4.5°C indicated a slower and more prolonged response with cortisol levels increasing 24 h after injection and persisting, in the case of LPS, for up to 48 h (data not shown). In contrast, the IL-1 β peptides P1 and P3 did not appear to influence the release of plasma cortisol when administered alone or in combination (Fig. 2a). Plasma glucose levels were significantly higher at 8 h than at 0 h in every treatment group ($P<0.001$ – $P<0.01$; Figs 1b and 2b), although there were no significant differences between treatments and their respective vehicle controls at any time point. However, there was a tendency for plasma glucose levels at 8 h in all treatment groups exposed to the REB vehicle (Fig. 1b) to be higher than the other treatment groups at 8 h (Fig. 2b). This difference was evident when comparing plasma glucose levels in PBS-treated fish with those of REB-treated fish ($P<0.05$; Figs 1b and 2b).

Confirmation that the dose of DEX selected for these studies was appropriate was provided by the confinement experiment. Plasma cortisol levels were not elevated (3.2 ± 1.2 ng/ml) in trout subjected to a confinement stressor after administration of DEX. However, plasma cortisol levels were elevated significantly following confinement (60 ± 8.9 ng/ml) in fish receiving a vehicle injection only. With respect to rIL-1 β and LPS, plasma cortisol levels were significantly and uniformly lower in fish receiving DEX compared with those that received DEX vehicle injection, at all sample times and for both LPS and rIL-1 β treatment (Fig. 3a). In strong contrast, the administration of rIL-1 β to DEX vehicle controls resulted in a significant elevation of plasma cortisol levels at 3, 6 and 8 h post-injection. Similarly, the administration of LPS elevated plasma cortisol levels in DEX vehicle-treated fish between 3 and 6 h although, in this experiment, no significant difference between LPS treatment and its control was evident at 8 h (Fig. 3a). Plasma glucose levels

Figure 1 The time course of changes in (a) plasma cortisol and (b) plasma glucose occurring in fish receiving a 400 μ l i.p. injection of either REB or rIL-1 β (0.1 or 0.6 nmol/kg). Fish were sampled immediately before injection and at 8–72 h thereafter. Each data set is represented by means $\pm$ S.E.M., $n=8$. Significant differences between the cortisol levels in REB and rIL-1 β (0.6 nmol/kg) at 8 h and between the glucose levels from 0 to 8 h are denoted by * $P<0.02$ and ** $P<0.001$ respectively.

in DEX- or DEX vehicle-treated fish showed no treatment-related alterations (Fig. 3b). However, overall, plasma glucose levels were significantly higher in DEX-treated fish than in DEX vehicle-treated fish ($P<0.001$).

Discussion

Until now, with respect to cytokines, the examination of immune–neuroendocrine interactions in fish has been

Figure 2 The time course of changes in (a) plasma cortisol and (b) plasma glucose occurring in fish receiving a 400 μ l i.p. injection of PBS, LPS (1.3 mg/kg), P1 (5.1 μ mol/kg), P3 (5.1 μ mol/kg) or P1/P3 (5.1 μ mol/kg). Fish were sampled immediately before injection and at 8–72 h thereafter. Each data set is represented by means $\pm$ S.E.M., $n=8$. Significant differences between the cortisol levels in PBS- and LPS-injected fish at 8 h and between the glucose levels from 0 to 8 h are denoted by * $P<0.05$ and ** $P<0.01$ respectively.

limited to the use of mammalian recombinant cytokines and cytokine antibodies (Balm *et al.* 1995, Ottaviani *et al.* 1995, Balm 1997). However, due to differences in sequence homologies, mammalian cytokine reagents may not necessarily behave in the same way (if at all) as the corresponding fish molecules, a premise exemplified by the absence of a cortisol response to murine IL-1 in tilapia (Balm *et al.* 1995, Balm 1997). Following the recent

Figure 3 The time course of changes in (a) plasma cortisol and (b) plasma glucose occurring in fish receiving a 400 μ l i.p. injection of PBS, LPS (1.3 mg/kg), REB or rIL-1 β (0.6 nmol/kg). Half of each treatment group was injected 24 h previously with either DEX (1 mg/kg in PBS:ethanol, 3:1; 150 μ l) or vehicle only. Fish were sampled 3–8 h after treatment injection. Each data set is represented by means $\pm$ S.E.M., $n=8$. Significant differences between experimental and respective control groups at each time point are denoted by * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

cloning of the trout IL-1 β gene and production of its recombinant protein, the present study, for the first time in fish, provides data that directly implicate IL-1 β in the activation of the HPI stress axis. In addition to cytokines,

bacterial endotoxins have been shown to indirectly elicit HPA activation through the systemic release of proinflammatory cytokines, including IL-1 β , IL-6 and tumour necrosis factor- α .

In fish research, LPS has been shown previously to activate the HPI axis *in vivo* through the detection of elevated plasma cortisol levels (Balm 1997). These observations, along with studies demonstrating the LPS-inducibility of the trout IL-1 β gene, highlight the suitability of LPS as a positive control molecule in the present study (Zou *et al.* 2000). The i.p. injection of either IL-1 β or LPS, at concentrations known to induce immunostimulatory effects *in vivo* (Hong 2002, Peddie 2002), evoked a rapid dose- and/or time-dependent stimulation of plasma cortisol levels, thus clearly implicating both molecules in the activation of the HPI axis. Furthermore, in terms of the time scale of the cortisol response to each treatment, the results are comparable with those of previous mammalian studies (Turnbull & Rivier 1999). However, fish are known to be relatively insensitive to LPS treatment, with concentrations required to stimulate immune and HPI responses greatly exceeding those required in mammalian models (Zou *et al.* 2000).

In addition to cortisol, a second widely used index of stress in fish has been the measurement of plasma glucose concentration (Pottinger & Carrick 1999). A wide range of stressors stimulate the sympathetic nervous system, leading to a rapid elevation of plasma glucose levels by a process mediated mainly by the glycogenolytic properties of circulating catecholamines (Wendelaar-Bonga 1997). Therefore, in light of the impact of rIL-1 β and LPS on cortisol levels, the effects (if any) of treatment on the concentration of plasma glucose were also investigated. Under the conditions employed in this study, there was no evidence of treatment-specific elevations in glucose levels normally associated with more general stress responses or any indication of hypoglycaemia as reported in similar murine studies (Rey & Besedovsky 1987). In support of these findings, subsequent mammalian studies have demonstrated that the peripheral administration of rIL-1 β may have no or only modest effects on the systemic release of catecholamines and hence on plasma glucose levels (Rivier *et al.* 1989). However, glucose levels were enhanced in all samples taken 8 h after treatment injection. Even though a stealth approach was taken to anaesthetizing fish prior to handling and injection, the catecholaminergic response is notoriously sensitive to disturbance and a release of catecholamines associated with anaesthetization, capture and handling may well account for the observed glucose increases. Furthermore, with respect to the DEX blockade, the potential gluconeogenic properties of DEX may account for the overall increase in glucose levels associated with DEX treatment relative to DEX vehicle controls.

To complement studies using the whole IL-1 β molecule, the neuroendocrine activity of two synthetic IL-1 β peptides (PI and P3) was also examined. The

first peptide, P1, corresponds to fragment 146–157 (YVTPVPIETEAR) of the trout sequence that is equivalent to the fragment 163–171 (VQGEESNDK) of the human IL-1 β sequence, known to be part of the receptor-binding domain (Antoni *et al.* 1986). The peptide P3 was synthesized to complex with an adjacent region of the IL-1 receptor, equivalent to fragment 207–216 (YRRNTGVDIS) of the trout sequence (Peddie *et al.* 2001). In humans, the 163–171 fragment induces a range of immunostimulatory events, although without the pyrogenicity and inflammatory effects normally associated with the whole molecule (Antoni *et al.* 1986). For the present study, an appropriate dose of P1 and P3 was provided by recent *in vivo* studies whereby P3 was shown to possess a range of immunostimulatory activities in trout (Hong 2002, Peddie 2002). Interestingly, however, at an equivalent dose no elevations in cortisol or glucose were observed following the injection of P3 alone or in combination with P1. In the context of using the whole rIL-1 β molecule to enhance the efficacy of fish vaccines, marked elevations in plasma cortisol may prove to be detrimental to the subsequent immune response. If following further research P3 is found to have no significant influence over CNS responses in trout, it may prove to be of value as an immune adjuvant in future fish vaccine development, as seen in mammals with respect to the 163–171 fragment of human IL-1 β (Beckers *et al.* 1993).

In mammals, it is generally accepted that the primary site of action of peripherally administered rIL-1 β is the hypothalamus. A direct effect of rIL-1 β on pituitary and adrenal tissues is believed to be too slow in onset to account for the rapid hormonal effects of this cytokine *in vivo* (Turnbull & Rivier 1999). Cytokine activation of the HPA axis is subject to feedback control exerted by circulating glucocorticoids. In this respect, the cortisol agonist DEX has been shown to specifically inhibit the IL-1 β -stimulated release of hypothalamic CRH and, hence, the subsequent secretion of ACTH and glucocorticoids (Loxley *et al.* 1993). In fish, DEX binds to specific corticosteroid receptors within brain tissues, including the hypothalamus (Allison & Omeljaniuk 1998). Subsequent studies have used DEX to block endogenous ACTH release without suppressing the cortisol-releasing capacity of the interrenal tissue (Pottinger & Carrick 2001). Therefore, DEX pretreatment was incorporated into the current study to further investigate the interaction of rIL-1 β and LPS with elements of the HPI axis. Consistent with mammalian studies, DEX completely eliminated the cortisol response to both rIL-1 β and LPS, thus supporting the premise that in fish IL-1 β and LPS affect plasma cortisol levels via interaction at the level of the hypothalamic–pituitary axis. Although the precise mechanisms of IL-1 β intervention within the CNS remain to be elucidated, the observed expression of a recently cloned trout IL-1 receptor type I-like gene in brain and pituitary cDNA samples provides further support for

a hypothalamic–pituitary site of action (JW Holland, unpublished observations).

Overall, the demonstration of the HPI-stimulatory activity of trout rIL-1 β in the present study, along with the future provision of other recombinant pro-inflammatory cytokines, will no doubt fuel research in the area of immune–neuroendocrine communication in fish.

Acknowledgements

This work was supported by a grant from NERC, UK (GR3/11695) and was performed under appropriate Home Office Project and Personal licences. Many thanks go to Mr Toby R Carrick (Centre for Ecology and Hydrology, Windermere) for his technical assistance throughout this study. There is no conflict of interest that would prejudice the impartiality of this manuscript.

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Received 15 April 2002

Accepted 12 June 2002