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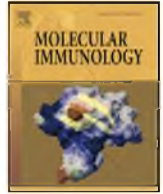
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Stress and innate immunity in carp: Corticosteroid receptors and pro-inflammatory cytokines

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ABSTRACT

The stress hormone cortisol is deeply involved in immune regulation in all vertebrates. Common carp (*Cyprinus carpio* L.) express four corticoid receptors that may modulate immune responses: three glucocorticoid receptors (GR); GR1, with two splice variants (GR1a and GR1b), GR2 and a single mineralocorticoid receptor (MR). All receptors are expressed as of 4 days post-fertilization and may thus play a critical role in development and functioning of the adult immune system. Immune tissues and cells predominantly express mRNA for GRs compared to mRNA for the MR. Three-dimensional protein structure modeling predicts, and transfection assays confirm that alternative splicing of GR1 does not influence the capacity to induce transcription of effector genes. When tested for cortisol activation, GR2 is the most sensitive corticoid receptor in carp, followed by the MR and GR1a and GR1b. Lipopolysaccharide (LPS) treatment of head kidney phagocytes quickly induces GR1 expression and inhibits GR2 expression. Cortisol treatment *in vivo* enhances GR1a and MR mRNA expression, but only mildly, and cortisol treatment *in vitro* does not affect receptor expression of phagocytes. Cortisol has no direct effect on the LPS-induced receptor profile. Therefore, an immune rather than a stress stimulus regulates GR expression. Cortisol administered at stress levels to phagocytes *in vitro* significantly inhibits LPS-induced expression of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF- α) and interleukin-12 (IL-12) (subunit p35) and of inducible nitric oxide synthase (iNOS) expression. A physiologically differential function for GR1 and GR2 in the immune response of fish to infection is indicated.

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1. Introduction

In teleostean fishes, such as carp, cortisol regulates a plethora of physiological processes such as development, reproduction, osmoregulation, metabolism, and immunity. Cortisol is the dominant steroid in stress physiology (Mommensen et al., 1999; Wendelaar Bonga, 1997) and has profound and differential effects on the immune system. Stress often inhibits immune responses, to counteract potential deleterious effects of too strong pro-inflammatory responses; acute stress and related cortisol release may exert stimulatory effects (Butts and Sternberg, 2008; Elenkov and Chrousos,

2006; Fast et al., 2007). Indeed, in common carp, stress reduces the number of circulating B-lymphocytes, and decreases the antibody response after immunization *in vivo* (Verburg-van Kemenade et al., 1999); cortisol inhibits inflammatory cytokine expression *in vitro* (Saeij et al., 2003c). Cortisol and lipopolysaccharide (LPS) synergistically stimulate the expression of interleukin 1 β (IL-1 β) mRNA in head kidney phagocytes (Engelsma et al., 2003). Interestingly, stress can have opposite effects. Cortisol stimulates apoptosis of B-cells (Weyts et al., 1998a), and inhibits apoptosis of neutrophils (Weyts et al., 1998b), an adaptive response to prolong the life span of neutrophils that form the first-line defence against pathogens.

Production in fish aquaculture is increasing rapidly over the last decades. Stress as a result of high rearing density and handling gives rise to immune suppression and increased susceptibility to infectious diseases, especially in larvae and juveniles (Maule and Schreck, 1991; Palermo et al., 2008; Terova et al., 2005). To steer disease prevention we need better understanding of mech-

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anisms of stress-induced modulation of immunity. In teleostean fishes, the stress hormone cortisol is produced following activation of the hypothalamus-pituitary-interrenal (HPI) axis, the functional analogue of the mammalian hypothalamus-pituitary-adrenal (HPA) axis. The hydrophobic cortisol enters the cell and activates cytosolic transcription factors, such as the glucocorticoid receptor. The hormone receptor complex translocates to the nucleus and binds to specific glucocorticoid responsive elements (GREs) in the DNA, to activate or repress transcription of genes (Kumar and Thompson, 2005). Formation of the ligand–receptor complex and transactivation or transrepression is hypothesized to utilise similar intracellular routes in teleostean fishes as in mammals (Stolte et al., 2006).

Teleostean fishes express more corticoid receptors than other vertebrates. Although fish do not produce aldosterone (Jiang et al., 1998), they do express mRNA and the protein of a mineralocorticoid receptor (MR), which can bind cortisol. Fish do produce significant amounts of 11-deoxycorticosterone (DOC) that may serve a role as mineralocorticoid, but physiological data on for instance plasma levels of this steroid in fishes are very scarce and await further studies (Prunet et al., 2006; Sturm et al., 2005). Furthermore, fishes have duplicate GR genes (GR1 and GR2) that both transcribe into functional proteins (Bury et al., 2003). Moreover, the GR1 gene may yield two splice variants, as was demonstrated for rainbow trout (Lethimonier et al., 2002; Takeo et al., 1996) and two pufferfish species (Stolte et al., 2006). Both splice variants are constitutively expressed and induce transcription (Greenwood et al., 2003; Takeo et al., 1996). Interestingly, the duplicated receptors require different concentrations of cortisol (low and high, basal and stress levels) to initiate transcription in effector cells (transactivation capacity) (Bury et al., 2003; Greenwood et al., 2003). This notion opens a vast range of opportunities for differential regulation with a single ligand, viz. cortisol.

The role of corticoid receptor subtypes in immune modulation was investigated. Corticoid receptor expression during early development suggests that stress or cortisol can affect the developing immune system. Based on constitutive mRNA expression levels and sensitivity for cortisol we show that the glucocorticoid receptors rather than the MR are important in immune regulation. GR expression is differentially adjusted following LPS treatment. We show inhibition of mRNA expression of the pro-inflammatory inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines by stress levels of cortisol. The results widen our insight into the intricate, cortisol-induced, immune modulation.

2. Experimental procedures

2.1. Animals

Common carp (*Cyprinus carpio* L.) were kept at 23 °C in recirculating UV-treated tap water at the “De Haar Vissen” facility in Wageningen. Fish were fed dry food pellets (Promivi, Rotterdam, The Netherlands) at a daily maintenance ration of 0.7% of their estimated body weight. The cross ‘R3 × R8’ is offspring of Hungarian (R8) and Polish (R3) strains (Irnazarow, 1995). Experimental repeats were performed with fish reared from different batches of eggs. All experiments were performed according to national legislation and were approved by the institutional Ethical Committee.

2.2. Tissue preparation

Nine month old carp (150–200 g) were anaesthetized with 0.2 g L⁻¹ tricaine methane sulphonate (TMS) (Crescent Research Chemicals, Phoenix, AZ, USA) buffered with 0.4 g L⁻¹ NaHCO₃

(Merck, Darmstadt, F.R. Germany). Blood was collected by puncture of the caudal vessels using a heparinized (Leo Pharmaceuticals Products, Ltd., Weesp, The Netherlands) syringe fitted with a 21-Gauge needle. Next fish were killed by spinal transection and organs and tissues were carefully removed, snap frozen in dry ice or liquid N₂ and stored at –80 °C for RNA extraction. Whole carp embryos were anaesthetized with 0.2 g L⁻¹ TMS buffered with 0.4 g L⁻¹ NaHCO₃ at the indicated stages of development. Individual eggs or embryos were snap frozen in liquid N₂ and stored at –80 °C.

2.3. Restraint–stress paradigm

Prolonged restraint (24 h) was given by netting the fish and suspending the nets with the fish in the tanks (Huising et al., 2004). After 24 h, the experimental group was transferred at once to a tank with 0.2 g L⁻¹ TMS, resulting in rapid (<1 min) and deep anaesthesia prior to blood sampling and killing. A control group was housed in an identical tank but left undisturbed. Control fish were sampled following rapid netting and anaesthesia, immediately before sampling of the experimental group. Blood and organs were isolated as mentioned above.

2.4. Plasma hormone determination

Freshly collected, heparinized blood was centrifuged for 10 min at 2000 × g at 4 °C, after which plasma was transferred to a new tube and stored at –20 °C. Cortisol was measured by RIA (Arends et al., 1998) with a commercial antiserum (Bioclinical Services Ltd., Cardiff, UK). All constituents were in phosphate-EDTA buffer (0.05 M Na₂HPO₄, 0.01 M Na₂EDTA, 0.003 M NaN₃, pH 7.4). Ten-microliter samples or standards in RIA buffer (phosphated-EDTA buffer containing 0.1% 8-anilina-1-naphthalene sulphonic acid and 0.1% (w/v) bovine γ-globulin) were incubated with 100 μL antiserum (in RIA buffer containing 0.2% normal rabbit serum) for 4 h. Samples were incubated overnight with 100 μL iodinated cortisol (around 1700 cpm/tube; ¹²⁵I-labeled cortisol, Amersham; Uppsala, Sweden) and 100 μL goat anti-rabbit γ-globulin (in RIA buffer). Bound and free cortisol in the assay were separated by the addition of 1 ml ice-cold precipitation buffer (phosphate-EDTA buffer containing 2% (w/v) bovine serum albumin and 5% (w/v) polyethylene glycol). Tubes were centrifuged at 4 °C (20 min at 2000 × g), the supernatant aspirated and the pellets counted in a gamma counter (1272 clinigamma, LKB, Turku, Finland).

2.5. Cell culture

Anterior head kidney phagocytes were obtained by passing the tissue through a 100 μm nylon mesh (BD Bioscience, Breda, the Netherlands) with carp RPMI (cRPMI, 280 mOsm) and washed twice. The cell suspension was layered on a discontinuous Percoll (Amersham, Biosciences, Uppsala, Sweden) gradient (1.020, 1.060, 1.070 and 1.083 g cm⁻³) and centrifuged 30 min at 800 × g with the brake disengaged. Cells at the 1.070 (65% macrophages, 10% granulocytes and 25% small macrophages and lymphocytes) and 1.083 g cm⁻³ (85% neutrophilic granulocytes, 15% macrophages) interface (Kemenade et al., 1994) were collected and washed twice with cRPMI and once with cRPMI⁺⁺ (cRPMI supplemented with 0.5% pooled carp serum, 1% glutamine (Cambrex, Verviers, Belgium), 1% penicillin G (Sigma–Aldrich, Zwijndrecht, the Netherlands) and 1% streptomycin sulphate (Sigma–Aldrich)). Subsequently treatments were carried out in duplo in cRPMI⁺⁺ at 5.5 × 10⁶ cells per well (in 500 μL) in a 24-well cell culture plate. Cells were stimulated for 4 h at 27 °C at 5% CO₂ with 30 μg ml⁻¹ LPS (Lipopolysaccharide form *Escherichia coli* 055:B5, Sigma–Aldrich), or with 100 nM corti-

Table 1
Primers used for gene expression studies (table shows sequences and Genbank accession numbers)

	FW primers	RV primers	Acc. No.
GR1a	GTCGTCGGATCAGCAAGC	CTGCGTTTTGTCGTCTCTC	AJ879149
GR1b	GTCGTCGGATCAGCAAGC	TTGTGCTGCCCTCTACG	AM697886
GR2	GGAGAACAACGCTGGGACTAAAT	GGCTGTCCCGATTAGGAA	AM183668
MR	TTCCCTGCAGAACTCAAAGGA	ACGGACGGTGACAGAAACG	AJ783704
IL-10	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGCTGTCA	AJ245635
TNF-α 1 and 2	GCTGTCGCTTACGCTCAA	CCTTGAAGTGACATTTGCTTTT	AJ311800
iNOS	AACAGGTCGAAAGGGAATCCA	CATTATCTCTCATGCCAGAGTCTCTCT	AJ242906
p35	TGCTTCTCTGCTCTGTGATGGA	CACAGCTGCAGTCTGTTCTGA	AJ580354
p40a	GAGCGCATCAACCTGACCAT	AGGATCGTGGATATGTGACCTCTAC	AJ621425
CXCa	CTGGGATTCCTGACCAITGGT	GTTGGCTCTCTGTTCAATGCA	AJ421443
CXCb	GGGACGGTGTITTTGTGTGA	AAGAGCGACTGCGGGTATG	AB082985
CXCR1	GCAAATTTGGTTAGCCTGGTGA	AGGCGACTCCACTGCACAA	AB010468
CXCR2	TATGTGCAAACGTATTTCAGGCTTAC	GCACACTATACCAACCATGTTG	AB010713
IL-10	CGCCAGCATAAAGAACTCGT	TGCCAAATACTGCTCGATGT	AB110780
TGF-β	ACGCTTTATTCCCAACCAAA	GAAATCCTTGCTCTGCCTCA	AAF22573
β-Actin	GCTATGTGGCTCTTGACTTCCA	CCGTCAGGCAGCTCATAGCT	M24113
40S	CCGTGGGTGACATCGTTACA	TCAGGACATGAACTCACTGTCT	AB012087

sol (Sigma-Aldrich) or a combination of both. Control cells received medium only and experiments were repeated for four independent fish. After stimulation, supernatant was removed and cells were collected in 300 µl RLT buffer from the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) and stored at -80 °C (duplicate treatments were pooled).

2.6. RNA isolation

RNA was isolated from tissues after extraction in Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was precipitated in iso-propanol, washed with 75% ethanol and dissolved in nuclease-free water. RNA of cells was isolated as described by the RNeasy Mini Kit (Qiagen) strictly according to the manufacturer's instructions. RNA concentrations were measured by spectrophotometry and integrity was ensured following electrophoresis in 1% agarose gel before proceeding with cDNA synthesis (Table 1).

2.7. DNase treatment and first strand cDNA synthesis

For each sample a '-RT' (non-reverse transcriptase) control was included. One microliter of 10× DNase-I reaction buffer and 1 µl DNase-I (Invitrogen, 18068-015) was added to 1 µg total RNA and incubated for 15 min at room temperature in a total volume of 10 µl. DNase I was inactivated with 1 µl 25 mM EDTA at 65 °C for 10 min. To each sample, 300 ng random hexamers (Invitrogen, 48190-011), 1 µl 10 mM dNTP mix, 4 µl 5× First Strand buffer (Invitrogen), 2 µl 0.1 M dithiothreitol (DTT) and 40 Units RNase Out (Invitrogen 10777-019) were added and the mix was incubated for 10 min at room temperature and for an additional 2 min at 37 °C. To each sample (not to the '-RT' controls) 200 U Superscript-II RNase H⁻ Reverse Transcriptase (RT; Invitrogen, 18064-014) was added and reactions were incubated for 50 min at 37 °C. Demineralized water was added to a final volume of 100 µl and samples were stored at -20 °C until further use.

2.8. Real-time quantitative PCR

PRIMER EXPRESS (Applied Biosystems, Foster City, CA, USA) and PRIMER3 software was used to design primers for use in real-time quantitative PCR (RQ-PCR) (Table 2). For RQ-PCR 5 µl cDNA and forward and reverse primers (300 nM each) were added to 7 µl Brilliant[®] SYBR[®] QPCR Master Mix (Stratagene, La Jolla, CA, USA) and demineralized water was added to a final volume of 14 µl. RQ-PCR (10 min 95 °C, 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C followed by 1 min at 60 °C) was car-

ried out on a Rotorgene 2000 real-time cycler (Corbett Research, Sydney, Australia). Raw data were analysed with comparative quantitation of the Rotor-gene Analysis Software V5.0. Basal gene expression in organs and tissues was determined as a ratio of target gene vs reference gene and was calculated according to the following equation: $ratio = (E_{reference})^{Ct_{reference}} / (E_{target})^{Ct_{target}}$ where E is the amplification efficiency and Ct is the number of PCR-cycles needed for the signal to exceed a predetermined threshold value. Expression following *in vitro* treatment was determined relative to the expression of non-restraint control fish according to the following equation (Pfaffl, 2001): $Ratio = (E_{target})^{Ct_{target} (control-sample)} / (E_{reference})^{Ct_{reference} (control-sample)}$. Two internal reference genes (40S ribosomal protein and β-actin) were incorporated in all RQ-PCR experiments; results were similar following standardisation to either gene. '-RT'-controls were included in all experiments and no amplification above background levels was observed. Non-template controls were included for each gene in each run and no amplification above background levels was observed. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The product of each template was checked by sequencing.

2.9. Transactivation assay

The clones encoding full-length open reading frame of common carp GR1a and GR1b were excized from pGEM-Teasy vector by EcoR1 and BamH1 and ligated into pcDNA3 expression vector, cut

Table 2
Cytokine mRNA expression of non-stimulated and LPS stimulated head kidney phagocytes

	Constitutive expression	LPS stimulated expression	Fold increase
IL-12 p35	6.69 ± 5.44 × 10 ⁻⁵	5.13 ± 4.97 × 10 ⁻⁴	7*
IL-12 p40	1.88 ± 1.28 × 10 ⁻⁴	2.07 ± 4.67 × 10 ⁻⁴	150*
CxGb	0.001 ± 0.001	0.002 ± 0.001	1
IL-10	0.004 ± 0.002	0.014 ± 0.007	4*
iNOS	0.046 ± 0.048	1.030 ± 0.331	75*
CxCR2	0.061 ± 0.034	0.077 ± 0.055	1
TNF-α	0.118 ± 0.119	0.350 ± 0.288	3*
TGF-β	0.212 ± 0.412	0.201 ± 0.376	1
CxCa	0.485 ± 0.180	0.422 ± 0.162	1
IL-1β	0.517 ± 0.320	3.436 ± 0.998	7*
CxCR1	0.709 ± 0.160	1.538 ± 0.328	2

Cells were stimulated for 4 h with 30 µg/ml LPS. Constitutive and LPS induced cytokine expression of four separate *in vitro* experiments as measured by quantitative real time PCR is shown relative to housekeeping gene 40S. Average-fold increase in cytokine expression as result of LPS treatment is mentioned.

with the same enzymes. Orientation and quality of the insert was confirmed by sequencing. COS-7 cells (derived from African green monkey kidney) were cultured as described previously (Sturm et al., 2005). Cells were transiently transfected using a calcium precipitation method (Sambrook and Russell, 2001), with cells growing in the log phase at 30–50% confluence. Cells were co-transfected with the following plasmids: expression vector with the appropriate hormone receptor cDNA (1 $\mu\text{g}/24\text{-well plate}$) reporter plasmid pFC31Luc, which contains the mouse mammary tumour virus promoter upstream of the luciferase gene (MMTV-LUC) (10 $\mu\text{g}/24\text{-well plate}$), and pSV β (Clontech, Palo Alto, CA, USA), a second reporter plasmid under control of the SV40 promoter and serving as a control for transfection efficiency (2 $\mu\text{g}/24\text{-well plate}$) and finally pBluescript (Clontech, Palo Alto, CA, USA) (7 $\mu\text{g}/24\text{-well plate}$), an irrelevant plasmid to increase transfection. Sixteen hr after transfection, medium was renewed and cortisol added from 1000-fold concentrated stock solution in ethanol. After 36 h incubation, cells were harvested using reporter lysis buffer (Promega, Madison, WI, USA) following the manufacturer's instructions and luciferase and β -galactosidase activities were determined as described previously (Bury et al., 2003). In addition to solvent controls (receiving only ethanol carrier instead of hormone) cells were transfected with an empty expression vector to control for luciferase activity in absence of hormone receptor DNA. Experiments were repeated three times independently with triplicate cell cultures per treatment. Luciferase activity was corrected for 'well-specific' transfection efficiency (determined by β -galactosidase activity) and then expressed as percentage luciferase activity observed in cells treated with 10^{-7} M cortisol. Kinetic parameters (maximum velocity, V_{max} and half maximum activation concentration (EC_{50}) in the transactivation assay were assessed by fitting the data to a single ligand binding model using SigmaPlot[®] software. Only converging data sets were included in data sets presented. Ligands were tested in the range of 10 pM to 1 μM . Data were normalized to maximal (100%) response and corrected for blanks prior to kinetic analysis.

2.10. Molecular modeling

Homology modeling techniques were used to construct a model of the carp glucocorticoid receptor (GR) DNA-binding domain (DBD). A crystal structure of the rat GR DBD in complex with DNA, solved at 2.5 Å resolution (Luisi et al., 1991), was used as a modeling template (Protein Data Bank (Berman et al., 2000) ID: 1R4O). Aside from the nine additional amino acids in the carp DBD, the sequences of the two DNA binding domains are virtually identical (98% sequence identity). The nine inserted amino acids were modeled using YASARA (<http://www.yasara.org>) by searching a non-redundant subset of the DBD for loops with similar sequence and start and end anchor points. Subsequently, side chains were modeled and the model was optimized using the Yamber2 force field to accommodate the changes (Krieger et al., 2004). A coordinate file of the model is available from the authors upon request.

2.11. Statistics

Statistical analyses were performed using SPSS 12.0.1 software. Differences in corticosteroid receptor expression were evaluated with a Student's *t*-test and $P < 0.05$ was accepted as fiducial limit. Homogeneity was tested with Levene's test and we corrected the Student's *t*-test for unequal variances when necessary. In case of RQ-PCR data, tests were performed for both internal reference genes (Actin and 40S) and statistical significance was only reported if both reference genes showed a significant effect. Cytokine expression and EC_{50} of receptors were compared and differences tested with a Mann–Witney U test, and $P < 0.05$ was accepted as sig-

nificant. Data are represented as average and error bars indicate standard deviation, *indicates $P < 0.05$, **indicates $P < 0.01$.

3. Results

3.1. Widespread mRNA expression of corticoid receptors in immune organs

GR1a, GR1b, GR2, and MR genes are constitutively expressed in all immune tissues and tissues rich in immune cells (epithelia of gills, skin gut and kidney) (Fig. 1A). The expression levels were comparable to those found in brain, hypothalamus and pituitary (Stolte et al., submitted) reported recently for the same species. Messenger RNA levels for the GR2 were consistently the highest, those for both GR1a and GR1b being about half this level and that for MR was on average less than 30% of GR2 expression. Gills and peripheral blood lymphocytes (PBL) showed the highest relative GR2 expression. In most organs tested GR1a expression was slightly higher than GR1b expression. MR expression levels were especially low in immune tissues; thymus, PBL, and head kidney.

During early development GR1 (a and b) mRNA expression levels in whole embryos were comparable to messenger RNA levels in separate organs of adult fish (Fig. 1B). In unfertilized eggs and embryos of 4 h post-fertilization (4 hpf) GR2 mRNA expression was ten-fold stronger than expression of either GR1 gene. These GR2 mRNA levels quickly and dramatically dropped until 24 hpf, after which levels remained constant. MR was hardly expressed until 24 hpf, but at 48 hpf expression levels started to rise, and at 96 hpf, MR mRNA expression levels were significantly increased compared to unfertilized eggs and 50% compared to expression of either GR1 gene.

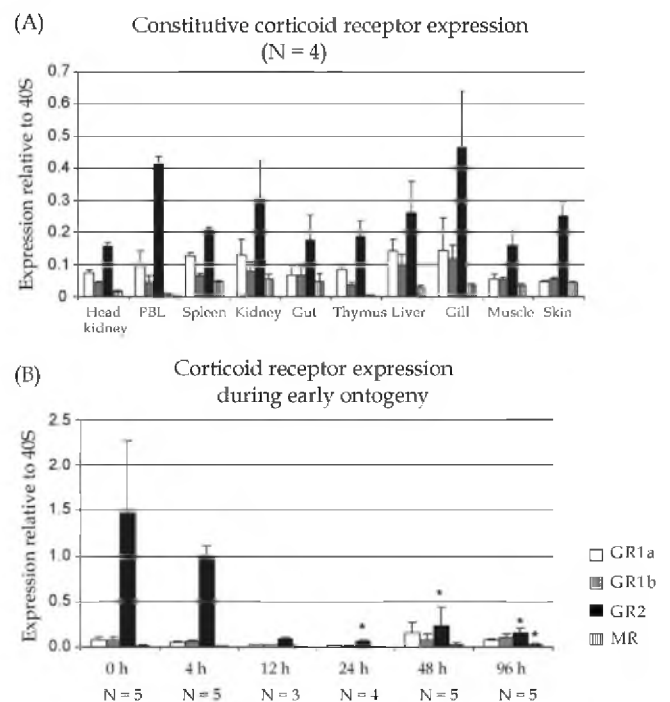


Fig. 1. Constitutive corticoid receptor mRNA expression in peripheral organs (A). cDNA of different organs, or freshly isolated blood lymphocytes, was used as template for quantitative real time PCR. Messenger RNA expression data of four control fish is shown relative to the housekeeping gene 40S. Corticoid receptor mRNA expression during early ontogeny (B). cDNA of four or five individual eggs or embryos (0, 4, 12, 24, 48 and 96 hpf) was used as template for quantitative real time PCR. Messenger RNA expression data is shown relative to the housekeeping gene 40S.

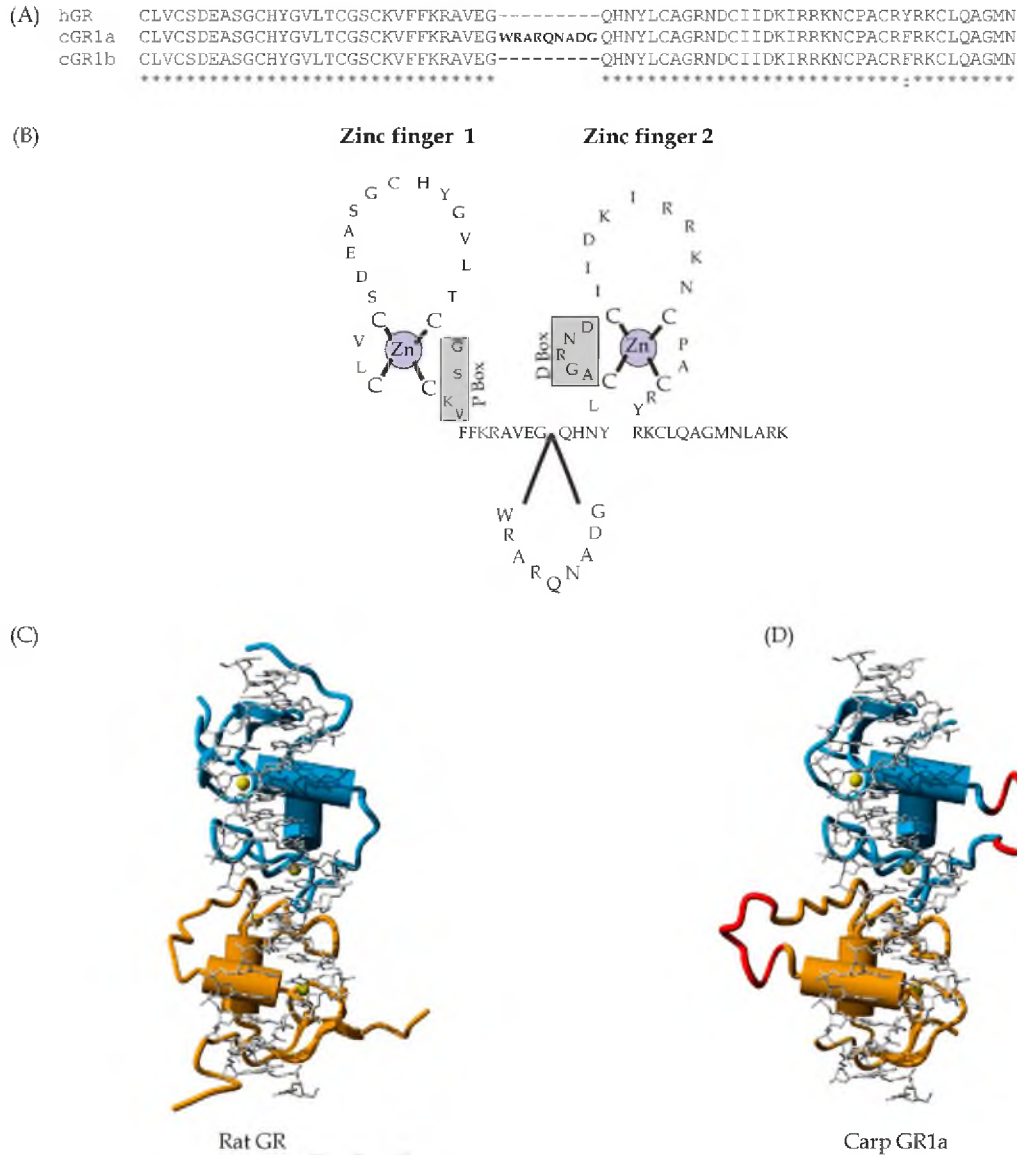


Fig. 2. Alternative splicing in the DNA binding domain of GR1. Schematic representation of the two zinc fingers in the GR DNA binding domain and location of insertion of 9 amino acids as a result of alternative splicing (A). Sequence alignment of rat GR (acc. number NP_036708) and common carp GR1a and GR1b. Identical amino acids are indicated by *, and amino acids with high and low similarity are indicated as : and -, respectively (B). Protein modeling of GR DNA binding region. DNA binding domain of carp GR1a (with 9 amino acid insert) is modeled over the rat GR crystal structure (PDB ID: 1R4O) (C). Wire frame represents DNA, ribbon and pipe structures represent two GR DNA binding regions that form a homodimer. Single balls represent zinc atoms of the zinc fingers. The 9 amino acid insert of common carp in each of the two DNA binding regions is indicated (D).

3.2. Homology modeling of the fish specific DNA binding domain

As the carp GR1a has a nine amino acid insert (WRARQNADG) in the very conserved DNA binding region (Fig. 2A and B), we first modeled the receptor DNA binding site. We constructed a protein model of the carp GR1a DNA binding domain based on the rat GR DNA binding domain crystal structure (Fig. 2C), and used the YASARA program to predict the three-dimensional structure of the nine amino acid insert. The nine amino acid insert extends the loop between the two zinc fingers and projects outward, away from the DNA (Fig. 2D). The model demonstrates that the insert does not necessarily disturb the zinc finger residues involved in DNA binding.

3.3. GR1a and GR1b transactivation

GR1a and GR1b showed similar affinities for the different hormones tested (Fig. 3); dexamethasone was the strongest ago-

nist tested, followed by cortisol, deoxycortisol and corticosterone. Aldosterone and DOC, were very weak agonists. The physiologically important stress hormone cortisol was chosen as ligand to compare sensitivity between the two splice variants. EC₅₀-values were found to be comparable; 7.1 ± 5.0 nM for GR1a and 17.4 ± 7.5 nM for GR1b, concentrations compatible with basal plasma cortisol levels.

3.4. Stress only mildly increases corticosteroid mRNA expression in head kidney

The effect of stress on corticoid receptor expression was determined in head kidney in head kidney *in vivo* and in head kidney phagocytes *in vitro*. In an *in vivo* experiment, 4 fish were confined in a net for 24 h, which resulted in acute stress, reflected by significantly increased plasma cortisol levels (Fig. 4A). After 24 h the head kidneys were removed and corticoid receptor mRNA expression was determined. GR1a and MR expression was slightly, but

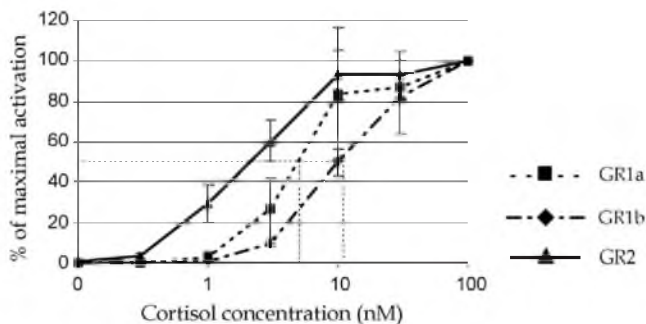


Fig. 3. Transactivation properties of glucocorticoid receptors in response to cortisol. COS7 cells were co-transfected with either GR1a (■), GR1b (◆) or GR2 (▲) expression vectors, together with reporter plasmid pFC31 Luciferase (under control of a MMTV promoter) and the pSVβ plasmid, which expresses β-galactosidase. After transfection cells were treated with varying (0.1–1000 nM) cortisol concentrations. Transactivation was determined by luciferase activity normalized to the internal β-galactosidase control. Data are expressed as percent activity of 100 nM cortisol and represent the average of three separate experiments. EC₅₀ is indicated by the dotted line.

sion was higher and constitutive MR expression was lower in head kidney phagocytes than in whole head kidney tissue. Cortisol did not significantly affect GR1 (a and b), GR2 and MR mRNA expression.

3.5. LPS treatment increases GR1a and decreases GR2 expression in head kidney phagocytes

Head kidney phagocytes were stimulated with 50 μg/ml LPS (Fig. 5A). GR1a expression was doubled after 2 h and was still increased after 4 h; GR2 expression was decreased at both 2 and 4 h. As immune stimuli often associate with a stress response, we determined *in vitro* the effect of a sub-optimal LPS stimulation (30 μg/ml) alone or in combination with 100 nM cortisol on corticoid receptor expression in head kidney phagocytes (Fig. 5B). Also, after 4 h of treatment with 30 μg/ml LPS, GR1 (a and b) mRNA expression was increased, while GR2 expression was decreased. After addition of cortisol to these LPS treated phagocytes, glucocorticoid receptor mRNA expression levels did not differ from expression levels after treatment with LPS only.

3.6. Cortisol inhibits LPS-induced iNOS mRNA expression

To mimick stress-induced immune modulation *in vitro*, the effect of cortisol exposure on head kidney phagocyte iNOS production in response to an immune stimulus (LPS) was investigated (Fig. 6). Compared to control cells, addition of 1 nM cortisol did

significantly increased; expression of GR1b and GR2 mRNA did not change significantly.

In a separate experiment the effect of 100 nM cortisol treatment on corticoid receptor expression in head kidney phagocytes *in vitro* was determined (Fig. 4B). Constitutive GR1 (a and b) mRNA expres-

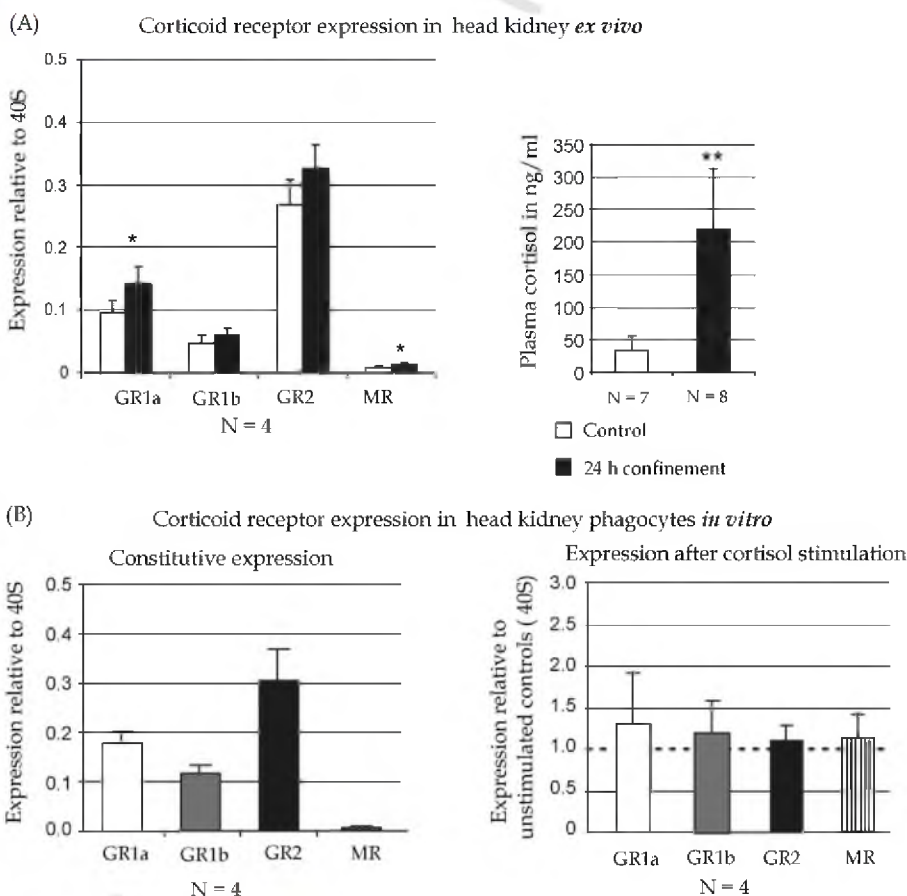


Fig. 4. Corticoid receptor mRNA expression after acute stress or *in vitro* cortisol treatment. After 24 h of confinement by netting, head kidneys from control or confined animals were used as template for real time quantitative PCR. Messenger RNA expression data of four fish per group are shown relative to the housekeeping gene 40S. Plasma cortisol levels of control and confined animals are shown in insert (A). After 4 h *in vitro* treatment with 100 nM cortisol head kidney phagocytes were used as template for real time quantitative PCR. Constitutive mRNA expression in head kidney phagocytes of four control fish is shown relative to the housekeeping gene 40S (B, left). Messenger RNA expression after cortisol treatment is shown as x-fold increase compared to non-stimulated control cells (set at 1, represented by the dotted line), standardized for the housekeeping gene 40S (B, right).

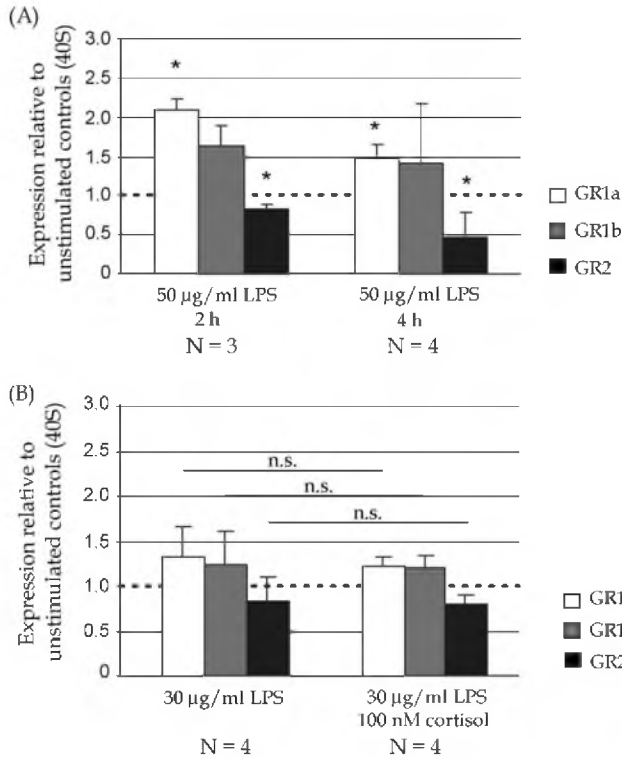


Fig. 5. LPS-induced glucocorticoid receptor expression. Freshly isolated head kidney phagocytes were stimulated for 2 or 4 h with 50 µg ml⁻¹ LPS. Messenger RNA expression data of four control fish is shown as x-fold increase compared to unstimulated control cells (set at 1, represented by the dotted line), standardized for the housekeeping gene 40S. Constitutive expression of control cells relative to the housekeeping gene 40S; 2-h treatment: GR1a: 0.022 ± 0.011, GR1b: 0.021 ± 0.010, GR2: 0.078 ± 0.036, 4-h treatment: GR1a: 0.028 ± 0.011, GR1b: 0.033 ± 0.018, GR2: 0.106 ± 0.005 (A). **Glucocorticoid** receptor expression after LPS or LPS and cortisol treatment. Freshly isolated head kidney phagocytes were stimulated for 4 h with 30 µg ml⁻¹ LPS or with 30 µg ml⁻¹ LPS and 100 nM cortisol. Messenger RNA expression data of four control fish are shown as x-fold increase compared to non-stimulated control cells (set at 1, represented by the dotted line), standardized for the housekeeping gene 40S. Constitutive expression of control cells relative to the housekeeping gene 40S; GR1a: 0.177 ± 0.025, GR1b: 0.118 ± 0.017, GR2: 0.306 ± 0.063 (B).

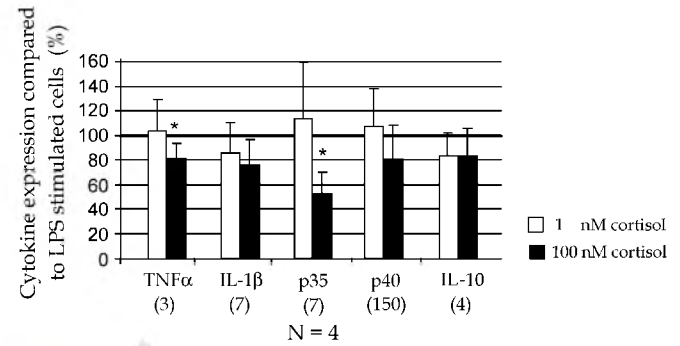


Fig. 7. Cytokine mRNA expression in LPS stimulated head kidney phagocytes. Freshly isolated head kidney phagocytes were stimulated for 4 h with 30 µg/ml LPS or a combination of LPS with either 1 nM or 100 nM cortisol. Data of four control fish are shown as percentage of LPS induced expression (set at 100%, represented by the thick line). Average increase of mRNA expression after LPS treatment is shown in (brackets).

not affect iNOS expression. Addition of 100 nM cortisol slightly decreased iNOS expression. Treatment with 30 µg/ml LPS increased iNOS expression 75-fold (12- to 200-fold) (Table 2). Addition of 1 nM (only GR2 activated) of cortisol did not affect LPS-induced iNOS expression, 100 nM cortisol (all GRs maximally activated) significantly decreased LPS induced iNOS expression.

3.7. Cortisol inhibits LPS-induced cytokine mRNA expression

Treatment with 30 µg/ml LPS significantly induced tumor necrosis factor alpha (TNF-α), IL-1β, interleukin 12 subunits p35 and p40 and interleukin 10 (IL-10) mRNA expression (Table 2). The same stimulus did not significantly affect mRNA expression levels of chemokines CxCa and CxCb, chemokine receptors CxCR1, CxCR2, and transforming growth factor beta (TGF-β) (Table 2). Addition of 100 nM of cortisol significantly inhibited LPS induced upregulation of tumor necrosis factor alpha (TNF-α) and interleukin 12 (IL-12) subunit p35 mRNA expression (Fig. 7). The lower concentration of 1 nM cortisol did not significantly affect expression levels of the cytokines measured.

4. Discussion

Common carp expresses four different corticoid receptors (GR1a, GR1b, GR2 and MR) and all four receptors are widely expressed in the central nervous system and pituitary gland (Stolte et al., 2008) as well as in peripheral organs. We here show differential roles for these glucocorticoid receptors (GRs) in cortisol-mediated immune modulation. We demonstrate constitutive expression of all corticosteroid receptors during development and in adult peripheral organs, their transactivation efficiency, and possible role in stress-related and immune modulation, as assessed by downregulation of expression of phagocyte pro-inflammatory mediators.

MR mRNA expression in typical immune tissues such as head kidney, peripheral blood lymphocytes (PBL) and thymus is very low. To better appreciate the functional consequences of this low constitutive expression it is important to know the transactivation capacity, i.e. the concentration of the natural ligand (cortisol) required to activate or repress an effector gene. Recent experiments (Stolte et al., 2008) have shown that GR2 is the most sensitive receptor (EC₅₀ 2.4 nM) whereas MR (EC₅₀ 4.0 nM) and GR1 (EC₅₀ 7.2 nM) are less sensitive. Considering the very low constitutive expression of the MR in immune organs and only moderate cortisol sensitivity we hypothesize that GRs rather than the MR are pre-

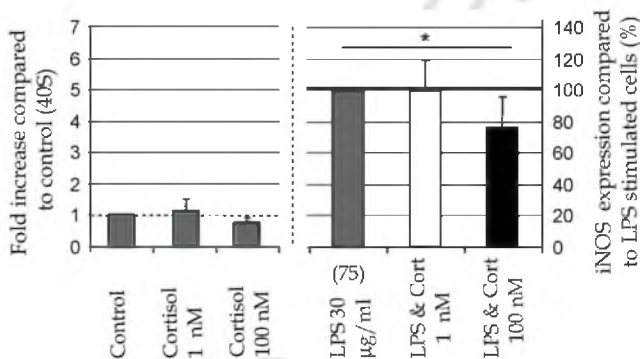


Fig. 6. iNOS mRNA expression in stimulated head kidney phagocytes. Freshly isolated head kidney phagocytes were stimulated for 4 h with either 1 nM or 100 nM cortisol, 30 µg/ml LPS or a combination of cortisol and LPS. Messenger RNA expression data of four control fish are shown as x-fold increase compared to non-stimulated control cells (set at 1, represented by the dotted line), standardized for the housekeeping gene 40S (left). Messenger RNA expression data of four control fish are shown as percentage of LPS induced expression (set at 100%, represented by the thick line) (right). Average increase of mRNA expression after LPS treatment is shown in (brackets).

dominantly involved in transmitting stress signals to the immune system.

Based on the widespread corticoid receptor expression in adult immune organs and our demonstration that corticosteroid receptor levels in the whole embryo are comparable to levels in separate organs of adult fish as from 4 dpf, it is tempting to hypothesize existence of cortisol-induced immune modulation at this early age. Alternatively, cortisol may play a role in the development of the immune system. Common carp show fast embryonic development of both the immune and the stress axis. After hatching at 2 days post fertilization (2 dpf) the larvae start feeding at 4 dpf when the yolk has been resorbed. Intriguingly, as early as 2 dpf endogenous ACTH and cortisol is produced and embryos show a 'stress response': whole body cortisol levels increase after handling (Sampath-Kumar et al., 1997; Stouthart et al., 1998; Flik et al., 2002). Around the same time (2 dpf), the developing immune system is capable of responding to an immune stimulus; LPS increased IL-1 β and iNOS expression (Huttenhuis et al., 2006). Since both the immune system and the stress axis are present around hatching, we assume that early life cortisol-induced immune modulation can affect the developing immune system.

The occurrence of two splice variants of GR1 in carp reminds strongly of the situation in rainbow trout (*Oncorhynchus mykiss*) and Burton's mouthbrooder (*Haplochromis burtoni*), and apparently such phenomenon is common in fishes (Greenwood et al., 2003; Stolte et al., 2006). Alternative splicing introduces 27 nucleotides in the DNA binding domain, which translate into a nine amino acid insert (WRARQNADG). Although this insert is conserved in evolutionary distantly related fish, rainbow trout and common carp are separated by 160 MY of evolution (Volf, 2005), other vertebrates do not show this insert (Stolte et al., 2006). However, other inserts at the same location in the DNA binding domain have been reported in both GR and MR of different vertebrate species (Bloem et al., 1995; Brandon et al., 1991; Rivers et al., 1999) and were shown to affect DNA binding and resulting transactivation capacity of the receptor (Wickert and Selbig, 2002). Human GR γ (Ray et al., 1996; Rivers et al., 1999) and the cotton top marmoset (*Saguinus oedipus*) GR (Brandon et al., 1991), show an extra Arginine (R), but its function was predicted to be comparable to wild type (Wickert and Selbig, 2002). Experiments, however, showed that although the transactivation capacity was unaffected, its V_{max} is diminished (Ray et al., 1996). Our model of the three-dimensional structure of the splice variant, based on the rat GR DNA binding domain crystal structure, predicts that addition of nine amino acids extends the loop after the interfinger alpha helix. This loop protrudes outside of the protein, and may thus be expected not to interfere with receptor DNA-binding. Our results corroborate a prediction for the rainbow trout GR-insert (WRARQNTDG) that also showed a loop extending to the outside of the protein (Wickert and Selbig, 2002). As both predictions were based on the DNA binding domain only, it remains uncertain if this prediction would hold for the entire protein. Transactivation experiments confirmed that the nine amino acid insert did not affect the capacity to activate a luciferase gene under control of a MMTV promoter. The EC_{50} 's are comparable for the variant with (GR1a) and without (GR1b) the insert and maximal activation was not altered. Also, EC_{50} -values for the potent synthetic steroid dexamethasone (GR1a 2.4 ± 3.8 nM and GR1b 2.9 ± 2.4 nM) were comparable as well (data not shown). Although these results show that alternative splicing does not affect DNA binding and resulting activation of transcription, the insert might affect interactions with co-activators and co-repressors (Kumar and Thompson, 2005). As the transactivation capacity for GR1a and GR1b are not significantly different, the mRNA expression levels of the variants combined can be summed and compared with GR2 expression levels. This results in similar relative expression levels in most immune related tis-

issues, except for PBL that show an overrepresentation of GR2 mRNA expression. Recently we forwarded the hypothesis of a 'sensitive' GR2 receptor that can induce transcription at basal cortisol levels, and an 'insensitive' GR1 receptor that requires stress levels of cortisol to induce transcription (Stolte et al., 2008). Our current data are consistent with this hypothesis.

To investigate differential roles of CRs in immune cells we determined receptor expression profiles after either an immune stimulus and/or a stress stimulus in head kidney phagocytes. In isolated head kidney phagocytes constitutive GR mRNA expression is higher and constitutive MR mRNA expression lower than in whole head kidney, which further augments the predominance of GR over MR in immune cells. Glucocorticoid receptor levels of head kidney phagocytes were slightly increased by treatment with stress levels (100 nM) of cortisol. Earlier results showed downregulation of GR binding sites in peripheral blood leukocytes of carp fed with cortisol-containing food (Weyts et al., 1998c), probably as a result of receptor translocation to the nucleus. In time, this might lead to increased mRNA levels to replenish GR numbers. Indeed, only after 24 h the increase in GR expression was significant. LPS treatment however, quickly induced GR1 mRNA expression, whereas GR2 expression was inhibited. This might reflect a temporal surge of the 'stress' GR1 receptor expression to increase sensitivity for feedback control as was shown for murine macrophages (Salkowski and Vogel, 1992). After LPS treatment, pro-inflammatory cytokine expression levels (IL-1 β , TNF- α , IL-12 (subunits p35 and p40) increase drastically with concomitant increase of nitric oxide (NO) (via inducible nitric oxide synthase) and toxic oxygen and nitrogen radicals directed to kill the invading pathogen (Engelsma et al., 2003; Huising et al., 2006; Saeij et al., 2003b; Saeij et al., 2000). This response is attenuated, likely to prevent detrimental and possible lethal effects. Indeed, in mice with GR-deficient macrophages higher mortality is seen after LPS treatment (Bhattacharyya et al., 2007). Although fish do not suffer from septic shock, they do show a strong pro-inflammatory response with high production of oxygen and nitrogen radicals which requires a firm balance to ensure effective pathogen clearance and prevent damage to the host. In mammals GR activity mediates immune suppression through inhibition of transcription factors such as activator protein (AP-1) and nuclear factor kappa B (NF κ B) that regulate expression of cytokines, inflammatory enzymes and inflammatory receptors (Rhen and Cidlowski, 2005). As intracellular pathways are much conserved throughout the vertebrate lineage, we predict the same inhibitory mechanisms for fish. Indeed NO production (under control of the inflammatory iNOS) was significantly decreased after treatment with a NF κ B inhibitor (Saeij et al., 2003a). LPS-induced expression of pro-inflammatory cytokine IL-1 β could be blocked by NF κ B inhibition (Engelsma et al., 2003).

To assess which CRs mediated immune modulation in common carp, we determined the ability of cortisol to affect LPS induced cytokine production. Basal plasma cortisol levels in carp are below 20 ng/ml and can increase to well over 200 ng/ml during stress. Roughly 80% of cortisol in circulation is bound to plasma proteins, which leaves a mere 20% of the total free and bio-active (Flik and Perry, 1989). With 1 nM (~2 ng/ml in plasma) and 100 nM (~200 ng/ml in plasma) cortisol in our cultures we could thus discriminate the cortisol concentration required to induce transcription by the GRs tested. At 1 nM cortisol, only the sensitive GR2 is activated, at 100 nM all three GRs will become maximally activated. The requirement of high levels of cortisol to induce inhibition of cytokine expression is in accordance with results of Saeij et al. (2003c), for IL- β , TNF- α and iNOS expression in head kidney phagocytes stimulated with lysate of the blood parasite *Trypanoplasma borreli*. In head kidney phagocytes constitutive expression of GR1 (combined) and GR2 are similar, and these levels were not differen-

tially regulated during our experiments. Despite the fact that high levels are necessary to initiate cytokine downregulation, not all immune modulation requires high levels of cortisol to take effect. Indeed, lymphocytes and especially B-lymphocytes, show high sensitivity for cortisol; significant inhibition of PBL proliferation and induction of apoptosis was measured at cortisol levels as low as 3.6 ng/ml, which can be due to activation of GR2 (Weyts et al., 1997). The strong over-representation of GR2 over combined GR1 expression in PBL suggests a role for cortisol in immune functions in non-stressed fish as well.

In conclusion differential gene expression of the duplicate GRs or splice variants is found in endocrine as well as in immune organs and cell types. The immune modulatory response of these receptors appears physiologically important, they are not only steered by endocrine signalling as an immune stimulus strongly and differentially regulates their expression profiles in leukocytes.

5. Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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