Cortisol stimulates the zinc signaling pathway and expression of metallothioneins and ZnT1 in rainbow trout gill epithelial cells

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Bury NR, Chung MJ, Sturm A, Walker PA, Hogstrand C. Cortisol stimulates the zinc signaling pathway and expression of metallothioneins and ZnT1 in rainbow trout gill epithelial cells. Am J Physiol Regul Integr Comp Physiol 294: R623–R629, 2008. First published December 12, 2007; doi:10.1152/ajpregu.00646.2007.—Intracellular zinc signaling is important in the control of a number of cellular processes. Hormonal factors that regulate cellular zinc influx and initiate zinc signals are poorly understood. The present study investigates the possibility for cross talk between the glucocorticoid and zinc signaling pathways in cultured rainbow trout gill epithelial cells. The rainbow trout metallothionein A (MTA) gene possesses a putative glucocorticoid response element and multiple metal response elements 1042 base pairs upstream of the start codon, whereas metallothionein B (MTB) and zinc transporter-I (ZnT1) have multiple metal response elements but no glucocorticoid response elements in this region. Cortisol increased MTA, MTB, and ZnT1 gene expression, and this stimulation was enhanced if cells were treated with cortisol together with zinc. Cells treated with zinc showed increased zinc accumulation, transepithelial zinc influx (apical to basolateral), and intracellular labile zinc concentrations. These responses were also significantly enhanced in cells pretreated with cortisol and zinc. The cortisol-mediated effects were blocked by the glucocorticoid receptor (GR) antagonist RU-486, indicating mediation via a GR. In reporter gene assays, zinc stimulated MTA promoter activity, whereas cortisol did not. Furthermore, cortisol significantly reduced zinc-stimulated MTA promoter activity in cells expressing exogenous rainbow trout GR. These results demonstrate that cortisol enhances cellular zinc uptake, which in turn stimulates expression of MTA, MTB, and ZnT1 genes.

glucocorticoid receptor; metal-regulatory transcription factor-I; fish; metals; glucocorticoid response element

METALLOTHIONEINS (MT) are small cysteine-rich metal-binding proteins that in mammals exist as four major isoforms: MT-I, -II, -III, and -IV (53). MT-I and MT-II constitute metal-inducible intracellular proteins that are expressed in most cells. In contrast, MT-III and MT-IV are not metal inducible and are expressed specifically in the central nervous system and squamous epithelia, respectively (40, 51). The biological functions of MT-I and MT-II include buffering of intracellular zinc, protection against metal toxicity (38, 43), and defense against oxidative stress (6, 12). The MTs found in different fish species are homologous to the mammalian MT and are functionally equivalent to the MT-I and MT-II isoforms (31).

The induction of MT expression by zinc is coordinated by metal-regulatory transcription factor-I (MTF1; Ref. 17). MTF1 is evolutionary conserved, and orthologues are present in Drosophila melanogaster, fish, mice, and humans (3, 8, 15, 56). Zinc causes MTF1 to translocate to the nucleus (47, 48) and enhances MTF1 binding to metal response elements (MRE) of target genes, including MT (2). Similar to the situation in mammals, MT genes in fish and many other species characteristically possess multiple MRE that drive MT transcription in response to MTF1 binding (7, 31, 37, 41). MTF1 also enhances expression of a growing list of genes involved in zinc homeostasis and responses to metal toxicity and oxidative stress (1, 47). A central theme to the mechanism of MT induction appears to be the ability of MTF1 to act as a sensor for labile intracellular zinc (2).

Transcription factors other than MTF1 are known to contribute to MT promoter activation in various species. There is conclusive evidence that glucocorticoids can induce MT expression in humans and mice independent of other gene activation factors (15, 28, 29). The response to glucocorticoids on MT expression in fish, on the other hand, is controversial. In vivo studies have shown that different types of stress (confinement, salinity exposure) can induce expression of MT or MT-like proteins in the liver of various fish species (11, 34, 44). Furthermore, treatment of cultured primary rainbow trout hepatocyte cultures with a supraphysiological concentration of cortisol (10 μM) has been shown to stimulate MT expression (36). In contrast, MT expression in the rainbow trout hepatoma (RTH-149) cells is unresponsive to cortisol treatment (36). Similarly, HepG2 and HEK-293 cells transfected with a reporter gene driven by the crucian carp MT promoter, which has two consensus glucocorticoid response elements (GREs), were unaffected by the synthetic corticosteroid dexamethasone (41). Given the equivocal data on cortisol-mediated MT expression in teleost fish, we hypothesized that cortisol-induced MT expression may not be directly regulated via glucocorticoid receptor (GR) binding to the GRE in the promoter region of the MT gene but via interaction between the glucocorticoid and zinc signaling pathways. In this scenario, cortisol would cause an increase in the intracellular concentration of labile zinc, triggering MT expression via the classical MTF1/MRE pathway.

In rainbow trout, there are two MT isoforms, MTA and MTB (7), which both possess tandem MREs located within 1–100 bp of the transcription initiation site and which are especially important for metal inducibility of the genes (33, 37, 45, 46). Rainbow trout MTA and MTB also have distal MRE within 1–100 bp of the transcription initiation site and which are characteristic of teleost fish MT promoters (21, 31, 41, 50). However, there are also several differing cis-acting elements in their 5’-flanking regions (Refs. 37, 45; Fig. 1); importantly for this study, the MTA promoter region possesses a GRE, whereas that of MTB.
does not. Thus cortisol may be expected to induce expression of MTA but not of MTB. In contrast, if cortisol stimulates MT expression via the zinc signaling pathway, then the expression of both MT isoforms will be expected to increase, as well as the expression of other MTF1/MRE-driven genes. Consequently, in addition to MTA and MTB, we measured expression of the gene encoding for solute carrier 30a1 (slc30a1), also known as zinc transporter-1 (ZnT1) (4), whose expression via the zinc signaling pathway, then the expression of MTA but not of MTB. In contrast, if cortisol stimulates MT expression, then the expression of MTA and MTB is decreased, and the gill cells were isolated according to the procedure described by Olsson and colleagues (37) and Samson and Gedamu (45). Putative transcription binding sites are shown for IL-6 (NF-IL-6), glucocorticoid response element (GRE), antioxidant response element (ARE), activation protein-1 (API), and metal-regulatory transcription factor-1 (MTF1; a metal response element (MRE)).

### MATERIALS AND METHODS

**Chemicals.** FBS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolum bromide (MTT), NaCl, KCl, NaHPO4, KH2PO4, ZnSO4·7H2O, HEPES, EDTA, hydrocortisone, mifepristone (RU-486), DMEM nutrient mixture F-12 Ham-Bradford reagent, and TRI reagent LS were purchased from Sigma-Aldrich. DMEM was purchased from Invitrogen. Leibovitz (L-15) medium with l-glutamine and phenol red, penicillin, and streptomycin, fungizone reagent, gentamycin reagent solution liquid, and trypsin-EDTA were obtained from Gibco BRL. Cell strainers (100 μm; Falcon), 0.4-μm cell inserts (12 well; Falcon), tissue culture insert companions (12 well; Falcon), and tissue culture plate (6 well) were purchased from Marathon. Powserscript reverse transcriptase and the plasmid pSV cDNA from Clonetech. RNase-free glycerogen, DNase I oligo(T)15, primer and random hexamers, luciferase assay kit, and the pGL2-Base plasmid were sourced from Promega. Gene-specific probes were from Qiagen. Pico green and FluoZin-3 were purchased from Marooon. Powerscript reverse transcriptase with oligo(dT)15 primer and random hexamers, following the manufacturer’s guidelines. Quantitative real-time PCR amplification was carried out on an ABI Prism 7000 sequence detection system (Applied Biosystems) using the following specific rainbow trout primers: 18S rRNA (GenBank accession no. AF-157514), with forward primer 5'-ggaggttggaagagcatca-3' and reverse primer 5'-tcgctagtggctttcgggt-3'; MTA (M18103), with forward primer 5'-catgaagctttgaagaacca-3' and reverse primer 5'-gcagctgag- geacacttg-3'; MTB (M18104), with forward primer 5'-taacagtgtaatattgcttaactc-3' and reverse primer 5'-aagagecggttggattcaca-3' and ZnT1 (AY-742790), with forward primer 5'-aacaagccctctgag-3' and reverse primer 5'-gttacagcttgaatggctgt-3'. The gene-specific probes used were 5'-JOE atacagctgtgcac BHQ-3' for 18S rRNA, 5'-VIC tgtgcagctgctg MBG-3' for MTA, 5'-6-FAM tgttaaagggagac BHQ-3' for MTB, and 5'-6-FAM tgtaagcagagcta BHQ-3' for ZnT1 in a buffer containing 200 mM Tris-HCl (pH 8.3), 500 mM KCl, 40 mM MgCl2, and 10 μM ROX. All reactions were carried out in triplicate.

**Intracellular chelatable Zn**2+. Relative concentrations of intracellular chelatable Zn**2+** were measured in gill cells cultured on six-well plates. Cultured gill epithelia were pretreated with 1 μM cortisol, 100 μM ZnSO4, 1 μM RU-486, or combinations for 24 h. Cells kept in L-15 only served as controls. The cells were washed three times with PBS and then incubated with 3 μM of the zinc-specific probe Fluozin-3-AM in L-15 medium. After a 60-min incubation, the cells were washed three times with L-15 and then incubated for another 30 min in L-15 medium to complete the intracellular cleavage of the FluorZin-3-AM. One milliliter of 135 mM NaCl, 1.1 mM EGTA, and 20 mM HEPES, pH 7.5, was added, and the cells were scraped off the inserts and homogenized. The homogenate (50 μl) was diluted with 2.0 ml of the same buffer, and fluorescence was measured with a spectrophotometer (Perkin-Elmer, LS 50B) with excitation at 494 nm and emission at 516 nm. Intensity of fluorescence was normalized to protein concentration, as measured with Bradford protein assay. Intracellular zinc concentrations in treated gill epithelia were expressed as percentage of the control.

**Zinc transport experiments.** Unidirectional influx of zinc across the cultured gill epithelium and rate of zinc accumulation into the cells was measured radioisotopically by 65Zn. Cultured gill epithelia were pretreated for 20 h with L-15 only or medium with 1 μM cortisol (basolateral compartment), 100 μM ZnSO4 (apical compartment), or 1 μM cortisol (basolateral compartment) in combination with 100 μM ZnSO4 (apical compartment). The medium on the basolateral side was then replaced with 2.0 ml of fresh L-15 without additives, and the apical medium was replaced with 1.5 ml of synthetic moderately hard water, containing 100 μM ZnSO4 and 1.5 kBq/ml of 65Zn. Flux of zinc was allowed to progress for 40 min, after which 1.0-ml aliquots of the media on either side of the epithelium were withdrawn for radioactivity counting (LKB Wallac, 1282 Compgamma). There was no change in volumes of media on the two sides of the cultured gill cells during the 40-min flux experiment. The cells were finally lysed with 1.7 ml of 1 M NaOH, of which 5 μl were saved for protein analysis; the remaining lysate was counted for radioactivity to determine cellular accumulation of zinc. Unidirectional influx of zinc across the epithelium was calculated from the total 65Zn radioactivity on the basolateral side and the specific activity of 65Zn (Bq/mmol) in the apical compartment. Similarly, zinc accumulation rate was calculated from the total amount of radioactivity in the cells and normalized to protein content and the specific activity of 65Zn in the apical compartment.
Expression vector constructs. The expression plasmid pCMrtGR1 (previously called pCMrtGR) encoding rainbow trout corticosteroid receptor rtGR1 has been described elsewhere (16). The reporter plasmid pGL2MTA-Luc, in which luciferase expression is driven by the rainbow trout MTA promoter, was obtained as described by Olsson et al. (37). The plasmid pSVβ, in which galactosidase expression is under the control of the SV40 promoter, was used to control transfection efficiency, whereas pBlueScript SK(+) (X52325) was used as an irrelevant plasmid to top up DNA amounts.

Reporter gene transactivation assays. COS-7 cell culture and transfection protocol followed that described in Sturm et al. (52). Cells cultured in 12-well plates were transfected with 5 μg of pGL2MTA-Luc, 2 μg of pSVβ, 10 μg of pVBlueScript SK(+), and up to 5 μg of pCMrtGR1. If >5 μg of pCMrtGR1 were used, empty vector pCMV5 (AF-239249) was added to give the same total DNA concentration. Twelve hours after transfection, cells were washed with PBS, the medium was renewed, and cortisol (1 μM) and/or zinc sulfate (80 μM) was added from 1,000-fold concentrated stock solutions, which had been prepared in ethanol for cortisol and in sterile water for zinc sulfate. After 30 h of incubation, cell extracts were analyzed for luciferase and β-galactosidase activities (22). If the reporter plasmid pGL2MTA-Luc was replaced by empty vector pGL2-Basic, luciferase activities was virtually absent in transfected cells regardless of treatment (data not shown). Experiments were repeated at least three times independently, with triplicate cell cultures per treatment. Luciferase activity was corrected for well-specific transfection efficiency as determined by β-galactosidase activity.

Statistical analysis. Data are expressed as means ± SD and are average values from three to nine values per experiment; experiments were repeated at least twice to confirm results. One-way ANOVA followed by Tukey’s test was used to compare the results from different treatments. For the transfection assays, results are expressed as a percentage of zinc-stimulated control (e.g., cells transfected with the pGL2MTA-Luc plasmid only and treated with zinc). Percentages were log transformed and assessed via a paired t-test. Statistical significance level was set to P < 0.05.

RESULTS

MTA, MTB, and ZnT1 gene expression in primary gill cell culture. A primary gill cell culture grown on semi-permeable supports was used to assess the specific effects of cortisol and zinc on MTA, MTB, and ZnT1 gene expression in rainbow trout. This gill cell culture system retains transepithelial resistance and polarity characteristics of the intact organ, enabling water to be placed on the apical side while the basolateral surface is bathed in L-15 medium (30, 54). When 5 μM zinc was added to the apical compartment, MTA and ZnT1 mRNA but not MTB mRNA were significantly increased compared with untreated cells (Fig. 2). With addition of 10 μM waterborne zinc, expression of all three genes was elevated compared with gene expression levels in control cells. Cortisol (applied basolaterally) induced expression of MTA, MTB, and ZnT1, but the genes showed different sensitivities to the hormone treatment. When 1 μM cortisol was added, resulting in plasma levels being equivalent to those recorded during severe stressful stimuli (55), all three genes displayed significantly increased expression above the untreated control (Fig. 2). With 0.25 μM cortisol, MTA and ZnT1 were significantly upregulated; with 0.1 μM cortisol, none of the genes investigated was upregulated. Combined treatment with 0.25 μM cortisol and either 5 or 10 μM waterborne zinc significantly enhanced expression of MTA, MTB, and ZnT1, at levels above those shown for untreated control and those for treatment with cortisol or zinc alone (Fig. 3). The enhanced effect of cortisol in combination with zinc on ZnT1 expression was likely mediated by the GR because it was abolished in the presence of the GR blocker RU-486 (Fig. 3).

Zinc influx and intracellular chelatable zinc. The positive effects of cortisol on the expression of MTB and ZnT1, neither of which have obvious GRE, -1000-bp and -2000-bp regions upstream of the start codon, respectively, led us to postulate that cortisol may increase intracellular zinc concentrations, in turn resulting in increased ZnT1 and MTB expression via the zinc-mediated MT1 signaling pathway. The fluorescent zinc probe FluoZin-3 (Kd = 15 nM) was used to determine the effects of cortisol and elevated external zinc levels on relative intracellular labile zinc concentrations. The cells were treated for 24 h with cortisol (1 μM), zinc (100 μM), or a combination, as well as in the presence or absence of RU-486 (1 μM), after which intracellular concentrations of FluoZin-3 chelatable zinc were determined. Cortisol alone, without any zinc present in the medium, did not affect the chelatable zinc concentration of the gill cells. In contrast, the addition of 100 μM zinc to the culture medium significantly increased the intracellular chelatable zinc concentration by 1.6-fold above results shown for control. Furthermore, the combination of 1 μM cortisol and 100 μM zinc substantially enhanced the intracellular labile zinc concentrations above those present in cells receiving the single treatments with cortisol or zinc. Again, it would appear that the cortisol component of this response was mediated by GR because addition of 1 μM RU-486 restored the intracellular chelatable zinc concentration back to the same level as that of the treatment with zinc alone (Fig. 4). These results suggest that cortisol had a stimulatory influence on the concentration of intracellular chelatable zinc, enabling it to rise when exogenous zinc was added to the system.

The increase in intracellular zinc could be due to either release of zinc from intracellular stores or an increase in uptake of zinc from the extracellular compartment. To assess the latter possibility, cellular accumulation rate and transepithelial influx of zinc (from the apical to the basolateral compartment), were measured over a 3-h period by the addition of 10 μM 65Zn to
the apical side and measuring the appearance of radiolabeled zinc in the cells or in the basolateral media. A 24-h pretreatment of cells with cortisol (1 μM) in the absence of zinc had no effect on either of the Zn²⁺ transport endpoints (Fig. 5). Pretreatment with 5 μM zinc resulted in a significant increase in transepithelial zinc influx, whereas combined treatment with zinc and cortisol further increased transepithelial zinc influx and also significantly increased the cellular zinc accumulation rate (Fig. 5).

Reporter gene transactivation activity. To more directly investigate the effects of zinc and cortisol, individually or in combination, on MTA expression, we used a reporter gene assay system in which COS-7 cells were cotransfected with the reporter construct pGL2MTA-Luc plasmid and in which firefly luciferase expression was driven by a 1,042-kb fragment of the rainbow trout MTA promoter region and differing amounts of rtGR1 (16) expression constructs. Previously reported experiments have shown that the rainbow trout corticosteroid receptors used are functional in COS-7 cells (10). In cells transfected with pGL2MTA along with rtGR1, zinc alone (80 μM) stimulated transactivation above the medium-alone control, demonstrating the functionality of the zinc-MTF1 pathway on the reporter gene construct (Fig. 6). In contrast, cortisol alone showed no transactivational activity (Fig. 6), suggesting that the -1042-kb MTA promoter, which has a single consensus GRE, is not activated by cortisol. Increasing the concentrations of the rtGR1 plasmid had no effect on zinc-induced transactivation compared with cells transfected with pGL2MTA-Luc alone. However, at a GR1 plasmid concentration of 1.5 and 5 μg per plate, transactivational activities in the presence of zinc and cortisol were significantly reduced compared with that shown in cells transfected with pGL2MTA-Luc alone and treated with zinc (P = 0.015 and 0.013). At 5 μg GR1, treatment with zinc and cortisol significantly reduced the zinc-induced transactivation activity (P = 0.008) (Fig. 6).

**DISCUSSION**

In the present study, we show that cortisol does increase MT expression in primary gill cell culture, in particular, when zinc is present in excess. Our results suggest that this stimulation is unlikely to be due to the direct effects of the cortisol-corticoid receptor complex acting as a ligand-inducible transcription factor and binding to the GRE present in the promoter region of the MTA gene but is a consequence of the cortisol-induced increase in the intracellular concentration of labile zinc. The elevated labile zinc activates the MTF1 signaling pathway, enhancing binding to MRE sites present in the promoter region of MT (47, 48). The hypothesis that cortisol induced MTA expression via activation of the MTF1 pathway is supported by the observation that both expression of ZnT1, a zinc exporter, and expression of trout MTB isoform gene increased in primary gill cell culture treated with cortisol. The 5'-flanking regions of ZnT1 and MTB do not possess any obvious GREs; however, similar to MTA, they are characterized by the presence of multiple MREs. The smaller effect of cortisol and zinc treatment on MTB expression compared with MTA expression may be explained by the presence of three
Furthermore, the retention of zinc in the perfused rat jejunum was found to be enhanced by dexamethasone administration or adrenalectomy, indicating a stimulatory effect on zinc absorbance or uptake (6). In the present study, we add to this information by showing that cortisol also increases transepithelial influx of zinc across an absorptive vertebrate epithelium, the gill, as well as the intracellular chelatable zinc concentrations.

To provide more mechanistic evidence as to whether cortisol can directly induce MTA promoter activity, a reporter plasmid (pGLMT-LUC) was constructed containing the −1042-bp 5′-flanking region of the MTA gene (37) linked to the firefly luciferase gene. Zinc treatment induced transactivation in COS-7 cells transiently transfected with the plasmid, demonstrating that the promoter was functional. In contrast, treatment with cortisol alone had no effect on MTA promoter activity in COS-7 cells cotransfected with pGLMT-LUC and plasmid expressing the recombinant rainbow trout GR1 (Ref. 16; Fig. 6). A similar result was obtained with the crucian carp MT promoter, which possesses a single GRE (41). However, the experiments with the MTA promoter revealed a curious result, in which zinc and cortisol treatment in combination appeared to reduce the MTA promoter activity in cells cotransfected with 5 μg of GR1 expression vector and the reporter plasmid (Fig. 6). The same result was obtained in cells transfected with the other rainbow trout corticosteroid receptors, rtGR2 or rtMR (10, 52), and treated in the same way (data not shown). This may suggest the presence of a negative GRE or a repressor function in this region. Indeed, a repressor of GRE function, which is responsive to stress, has been identified in the pro-

<Fig. 5. Primary gill cell culture cellular zinc accumulation (top) and transepithelial zinc influx (bottom) after a 20-h treatment with cortisol (1 μM), zinc (5 μM), or combination of zinc and cortisol or left untreated (Con). Cellular zinc accumulation and transepithelial influx were determined by the addition of 65Zn to the apical chamber for the last 40 min of the treatment. Values are average ± SE. Bars with different letters are significantly different from each other (ANOVA followed by a Tukey-Kramer post hoc test, P < 0.05, using untransformed data).

<Fig. 6. MTA promoter activity in COS-7 cells without addition of zinc or cortisol (control; solid black bars) or in the presence of cortisol (1 μM; light gray bars), zinc (80 μM; open bars), or zinc (80 μM) and cortisol (1 μM) in combination (hatched bars). COS-7 cells were transiently transfected with 5 μg of pGLMTA-Luc reporter plasmid, different concentrations (0, 0.5, 1.5, and 5 μg) of rainbow trout GR expression construct, 2 μg of β-galactosidase reporter (pSVβ, to correct for transfection efficiency), and pBluescript SK to top up DNA to 12 μg. Data are expressed as a percent of the luciferase activity measured in cells transfected with only 5 μg pGLMTA-Luc and treated with 80 μM zinc (positive control). Each bar represents average ± SE from 3 separate experiments. *Significant difference to the positive control, #significantly different from those cells transfected with 5 μg pGLMTA-Luc and no GR and treated with zinc and cortisol, and *significant difference between cells that have been transfected similarly but treated with either zinc or zinc and cortisol (paired t-test, P < 0.05 using log-transformed data).>
In conclusion, the present study demonstrates that cortisol-induced expression of MTA, MTB, and ZnT1 in fish gill cells is due to cortisol stimulation of zinc influx and subsequent activation of the zinc MTF1 pathway. The observation that cortisol can mediate zinc influx may be of significance in other cells, such as the lung and immune system, where cross talk between the GR and zinc signaling may play an important role in modifying the effects of gene expression.

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