Gene expression and TNF-alpha secretion profile in rainbow trout macrophages following exposures to copper and bacterial lipopolysaccharide

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Abstract

Fish macrophage function can be altered after exposure to pathogens as well as to xenobiotics. Considering that wild and farmed fish can be exposed in their habitats simultaneously to different types of stressors, including chemical contaminants (e.g. heavy metals) and pathogens (e.g. bacteria), it is fundamental to study their impact either isolated or in combination. Therefore, the present study aimed to evaluate the effects of copper and bacterial lipopolysaccharide (LPS), alone and in combination, on the transcription of target genes related with immune system, respiratory burst activity and cell death, using rainbow trout macrophages as in vitro model. A cell viability experiment was performed to determine the sub-lethal concentrations of copper for rainbow trout macrophages and the LC50-24 h was estimated at 60 mM. The expression of proinflammatory cytokines, such as interleukin-1b (IL-1b), interleukin-6 (IL-6) and tumour necrosis factor-a (TNFa) increased after copper and copper plus LPS exposure. Copper and LPS interact positively inducing an increase in cytokine expression, which may be indicative of an increased inflammatory response. However, the increase in TNFa mRNA expression induced by 50 mM copper was not accompanied by protein secretion indicating that mRNA abundance does not always reflect the level of protein and that the translation of the TNFa mRNA is somehow inhibited. Serum amyloid A (SAA) and trout C-polysaccharide binding protein (TCPBP) mRNA expression also increased after copper, LPS or LPS plus copper exposure, indicating a role of acute phase proteins in the local response to inflammation. NADPH oxidase and glutathione peroxidase gene expression increased in macrophages after 24 h exposure to copper, LPS or LPS plus copper. The results from the present study improve the understanding of mechanisms involved in copper toxicity, as well as the interaction with a simulated-inflammatory process.

1. Introduction

Macrophages are multifunctional cells of the immune system with extended functional roles ranging from the clearance of microorganisms, xenobiotic material and apoptotic cells to the regulation of both innate and acquired immune responses. They are involved in phagocytosis, antigen presentation and release of anti-microbial and anti-tumour agents, as well as in the inflammatory reaction by secreting cytokines and chemokines [1,2]. Proinflammatory cytokines, such as interleukin-1beta (IL-1b), interleukin-6 (IL-6) and tumour necrosis factor-a (TNFa), in turn, induce the synthesis of acute phase proteins (APP) [3], including serum amyloid A (SAA) which is considered, in mammals, a major APP due to its elevation in plasma in response to different inflammatory conditions [4]. In fish, as in mammals, SAA is mainly synthesized in hepatocytes [5] although its expression in extra-hepatic tissues has also been reported [6,7]. Trout C-polysaccharide binding protein (TCPBP) is also considered an APP [3], but little is known about its expression in fish and specifically in macrophages.

Studies on fish macrophages demonstrated that phagocytic and respiratory burst activity are activated by exposure to bacteria [8], bacterial lipopolysaccharide (LPS) [9], virus [10], and fungal cell wall components [11]. Parallel studies also reported that macrophage functions can be modulated by different classes of environmental contaminants [12,13]. The majority of studies conducted under each

Abbreviations: APP, Acute phase protein; GPx, glutathione peroxidase; HKM, head kidney macrophages; HMGB1, high mobility group box 1; IL-1b, interleukin 1 b; IL-6, interleukin 6; SAA, serum amyloid A; TCPBP, trout C-polysaccharide binding protein; TNFa, tumour necrosis factor-a.

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context have been focused on the response to single stimuli, immune or xenobiotic, and studies addressing the combined action of these two stressors are limited. Considering that wild and farmed fish may be simultaneously exposed to different types of stressors, including chemical contaminants (e.g. heavy metals), investigation of their impact on macrophage activation pathways during combined exposures is of relevance. Among heavy metals, copper is frequently found in aquatic systems, including freshwater bodies. Copper is an essential nutrient, but it becomes toxic at high concentrations, interfering with several metabolic pathways and thereby inducing different responses at cellular and higher levels [14]. Previous in vivo studies with fish revealed that copper induces changes in the transcription of genes related with the immune and cellular stress responses [15e17]. Nevertheless, these studies concerned the isolated effects of copper on the whole head kidney and, to our knowledge, no studies have been conducted assessing the effects of copper upon bacterial lipopolysaccharide-induced macrophage activation in fish.

It has been reported that copper toxicity is, in part, due to the over-generation of reactive oxygen species (ROS) and depletion of antioxidant defences [18]. On the other hand, ROS, showing a dual role, are produced by macrophages as cytotoxic agents against pathogens during the respiratory burst, via stimulation of the NADPH oxidase system. Divergent results were reported by Murphy et al. (1995) [19] demonstrating that oxidative stress may depress macrophage function, while Kirkham (2007) [20] stated that macrophages activated under conditions of oxidative stress can generate enhanced inflammatory responses.

The present study aimed to evaluate the responses at the level of gene expression including target mRNAs relevant to the activation of immune system (IL-1b, IL-6, TNFa), inflammation (SAA, TCPBP), cell death (caspase 6 and high mobility group box 1eHMGB1), respiratory burst activity (NADPH oxidase) and antioxidant activity (glutathione peroxidaseeGPx) of differentiated head kidney macrophages (HKM) to exposure to both copper and LPS, individually and in combination. Finally, as mRNA abundance may not strictly correlate with protein levels, the secretion of TNFa, was also assessed.

2. Materials and methods

2.1. Animals

Rainbow trout (Oncorhynchus mykiss) specimens, weighing approximately 170 g, were obtained from a local fish farm (Piscifactoria Andrés, St. Privat d'en Bas, Spain). Fish were acclimatized to laboratory conditions for at least 15 days, maintained in a closed freshwater flow circuit, at 15 °C, in a 12 h light/12 h dark cycle and fed a commercial diet once a day. Water quality indicators, such as dissolved oxygen, pH, nitrite and ammonia were analyzed periodically, and the measured values were acceptable considering rainbow trout physiological requirements.

All experimental procedures were submitted to the Ethical Committee of the Universidad Autonoma de Barcelona and authorized by the "Departament d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya", that agree with the International Guiding Principles for Biomedical Research Involving Animals.

2.2. Macrophage isolation and in vitro assays

Fish were sacrificed by over-anesthetization with tricaine methane sulphonate (MS222, SigmaeAldrich), bled from the caudal vein to remove as much blood as possible and head kidneys were excised. Cells from the head kidney were isolated and cultured as described previously [1]. Briefly, head kidneys were homogenized using 100 mM nylon mesh cell-strainers in presence of Dulbecco's Modified Eagle Medium (DMEM) containing high glucose, 10% heat inactivated fetal bovine serum gold (Labclinics, Spain) and the antibiotic Primocin (100 mg/ml) (Invivogen). DMEM is a metal-free culture medium. The homogenates were then centrifuged for 1 min at 1200 rpm, resuspended in 12 ml of medium and plated on 12-well poly-D-lysine (SigmaeAldrich) treated cell culture plates, 1 ml per well. The cultures were kept at 17 °C and 5% CO_2. Non-adhering cells were removed after 24 h and new medium was added. The adherent cells were incubated for another 4 days before treatment, changing the medium every 2 days. It was previously shown that head kidney cells incubated for 5 days under these conditions have the typical cytological characteristics of macrophages and an increasing phagocytic capacity and cytokine expression [1].

First, a cell viability experiment was performed in order to estimate the LC50-24 h for copper (concentration that kills 50% of macrophages after 24 h exposure) and to determine the concentrations to be used in the in vitro toxicity assay. For the copper and LPS treatments, the medium of each well was removed and fresh medium containing the required amounts of copper (as copper chloride, SigmaeAldrich), LPS from Escherichia coli (SigmaeAldrich) or LPS plus copper were added and the cultures incubated for 24 h. The tested concentrations were: 0.1, 1, 10, 50, 100, 1000 and 2000 mM copper, 10 mg/ml LPS or LPS (10 mg/ml) plus 1, 10 or 50 mM copper (the experiments were done using four fishes per treatment, n ¼ 4), and one well of each culture plate served as a control. After exposures, cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay (SigmaeAldrich). The MTT assay is based on the cellular uptake of MTT and its subsequent reduction in the mitochondria of living cells to MTT formazan. Dead cells do not cause this change. Following the 24 h exposure with copper, LPS and copper plus LPS, medium was discarded and each well washed with PBS (1x). After that, cells were incubated with 800 ml/well of DMEM plus 80 ml/well of MTT solution (5 mg/ml) for 1 h at 37 °C. The medium containing MTT was removed and the formazan crystals solubilised with dimethyl sulfoxide and absorbance read at 570 nm. Cell viability was expressed as a percentage of the control.

For the main experiment, cells were isolated as described previously but the homogenates were plated on 6-well poly-D-lysine treated plates (2 ml/well) and kept at 17 °C and 5% CO_2. Afterwards, the medium of each well was removed and fresh medium containing the required concentrations of copper (1, 10 or 50 mM Cu), LPS (10 mg/ml) or LPS (10 mg/ml) plus copper (1, 10 or 50 mM) was added and the cultures incubated for 24 h.

2.3. Analysis of gene expression

Total RNA was isolated from HKM using 1 ml/well of Tri Reagent (Molecular Research Center Inc.) following the manufacturer's instructions. Quantification was carried out with a Nanodrop1000 (Thermo Scientific) and the quality of the RNA was checked with a Bioanalyser (Agilent Technologies). All RIN values obtained were >9, indicative of excellent RNA integrity and quality. Five mg of RNA were used to synthesize cDNA with Superscript III™ reverse transcriptase (Invitrogen) and oligo-dT primer (Promega). After that, cDNA was used for quantitative polymerase chain reaction (RT-PCR) analysis using SYBR Green PCR Supermix (Bio-Rad). The sequences of the primers used in gene expression analysis are presented in Table 1. Total volume (20 ml) in every reaction contained 500 nM of each amplification primer, 10 ml of iQ™ SYBR Green Supermix (Bio-Rad) and 5 ml of a 1:50 dilution of cDNA (1:500 for 18S). Controls lacking cDNA and controls containing RNA were included. Reactions were run in the iCycler iQ™ Real-time PCR Detection System

Table 1
Sequences of primers used in gene expression analysis and accession numbers.

Gene	Forward primer	Reverse primer	Accession number
IL-1b	GGAACCGAGTTCAAGGACAA	GGCTACAGGTCTGGCTTCAG	NM_001124347.1
TNFa	CGCTGACACAGTGCAGTGGA	TCCCCGATGGAGTCCGAATA	AJ401677
IL-6	TTTCAGAAGCCCGTGGAAGAGA	TCTTTGACCAGCCCTATCAGCA	DQ866150
SAA	GGGAGATGATTCAGGGTTCCA	TTACGTCCCCAGTGGTTAGC	X99387
TCPBP	TCCAGGACAATGAGGGCTAC	ACTGTGGACCAGGTTGGTGT	AF281345
NADPHoxid.	CATCGCCCACCTGTTTAACT	GTATGACCTGCGGATGACCT	AB192465.1
GPx	GATTCGTTCCAAACTTCCTGCTA	GCTCCCAGAACAGCCTGTTG	BG934453
HMGB1	CTTCAGCTTTGCAGCCTT	AAGATCCAAGGAAGCCGA	DQ403261
Caspase 6	TGGTCTACACCCTCCCTGCT	GCTCCTCATGGACACCTTCC	AF212219
18S	CGAGCAATAACAGGTCTGTG	GGGCAGGGACTTAATCAA	AF243428.2

(Bio-Rad Laboratories), under the protocol: 1 cycle of 95 °C for 5 min, 40 cycles of 95 °C for 10 s and 60 °C for 30 s, 1 cycle of 95 °C for 1 min, 1 cycle of 55 °C for 1 min, and a melting curve of 81 cycles (from 55 °C to 95 °C). All samples were run in triplicate. C_T (threshold cycle) values for each sample were expressed as "fold differences", calculated relative to untreated controls and normalized to endogenous control 18S rRNA. Throughout all experimentation Ct values for 18S rRNA in the RTQ-PCR assays were observed in the same range (18e22 cycles) as in all other experiments that we have carried out in this cell type.

2.4. Western blot analysis of supernatants

Supernatants from stimulated cell cultures were recovered, centrifuged and stored at −80 °C until use. Supernatants were boiled in the presence of Laemmli buffer for 5 min and loaded onto 15% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Immmobilion, Millipore) using a Miniprotean III system (Bio-Rad). Before blocking, membranes were stained with Ponceau-S (Sigma) and completely "faded" with Towbin buffer and washed with TBST before blocking for 2 h at room temperature in TBST with 5% non-fat dry milk. For TNFa detection, membranes were incubated with anti-TNFa antibody 1:500 in blocking buffer overnight at 4 °C and after 3 washes with TBST; the membrane was incubated with Goat Anti-Rabbit IgG, HRP conjugated-antibody (Jackson Immuno Research) 1:5000 in blocking buffer for 2 h at room temperature. The membranes were developed using the enhanced chemiluminescence detection kit (SuperSignal West Pico substrate, Pierce) visualized and quantified with an image analyzer (ChemiDoc with QuantityOne software, BioRad).

2.5. Data analysis

A logarithmic regression was adopted to estimate the LC50-24 h. Statistica software (StatSoft Inc., Tulsa, OK) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. Differences between control and exposed groups were tested using one way ANOVA, followed by LSD post hoc test and the significant level was ascertained at $P < 0.05$ for the preliminary experiment (viability). For the main experiment, a factorial ANOVA was used to compare results between groups and evaluate eventual interactions, followed by LSD test. Significance of results was ascertained at $P < 0.05$.

3. Results

Rainbow trout HKM incubated during 24 h with copper displayed cellular viability above 75%, in relation to the control, at concentrations up to 10 mM (Fig. 1). For concentrations higher than 10 mM, cell viability decreased in a concentration-dependent manner, reaching values near 100% mortality at 2000 mM. All tested concentrations of copper caused a statistically significant reduction in HKM viability when compared to control. The LC50-24 h for copper was estimated at 60 mM (R^2 ¼ 0.93 and $p < 0.001$). HKM incubated with only LPS (10 mg/ml) had a viability score of 95.6%. Concerning the effect of mixtures, HKM exposed to LPS plus 1 mM copper had a viability of 84%, a significant decrease when compared to control. LPS plus 10 or 50 mM copper also induced a significant decrease compared to control and to LPS alone; however, cell viability was above 50% in both cases.

Exposure of HKM to copper caused a general elevation on cytokine mRNA abundance when compared to control values (Fig. 2Ae C). IL-6 expression was significantly increased 7-, 8- and 9-fold after incubation with 1, 10 and 50 mM copper, respectively, displaying a dose-dependence (Fig. 2C). However, IL-1b and TNFa expression were significantly increased only after incubation with 50 mM copper (86 and 55-fold, respectively) (Fig. 2A and B). LPS exposure caused an increase in mRNA abundance for all cytokines, though only significant for IL-6 (Fig. 2C).

Exposure of HKM to LPS plus copper (1, 10 and 50 mM) caused a significant elevation in expression of all cytokines when compared to control. Additionally, exposure of HKM to all the mixtures caused an increase in IL-1b and TNFa expression when compared to LPS. For these two cytokines, the increase induced by LPS plus 1 or 10 mM copper was significantly higher than the increase induced by copper alone. LPS plus 1 or 10 mM copper induced a potentiation in both IL-1b and TNFa mRNA expression.

LPS plus 1 mM copper caused an increase in IL-6 transcription greater than the increase caused by 1 mM copper alone. Furthermore, LPS plus 10 and 50 mM copper caused an increase of IL-6 compared to LPS, and to the respective concentrations of copper alone, pointing out a synergistic interaction.

Copper exposure did not induce the secretion of TNFa from the cells to the supernatant. However, LPS and LPS plus all copper concentrations caused an increase in the secretion of this protein in

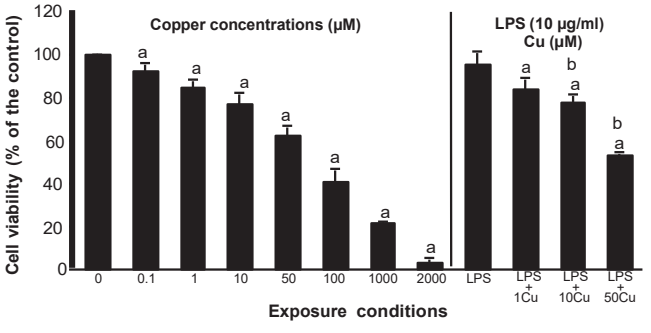

Fig. 1. Viability (expressed as a percentage of the control) of rainbow trout macrophages exposed to copper (0.1 mMe2000 mM), LPS (10 mg/ml) or LPS (10 mg/ml) plus copper (1, 10 and 50 mM) during 24 h. Values represent the means and S.E. (n ¼ 4). Significant differences are: a vs. control; b vs. LPS ($P < 0.05$).

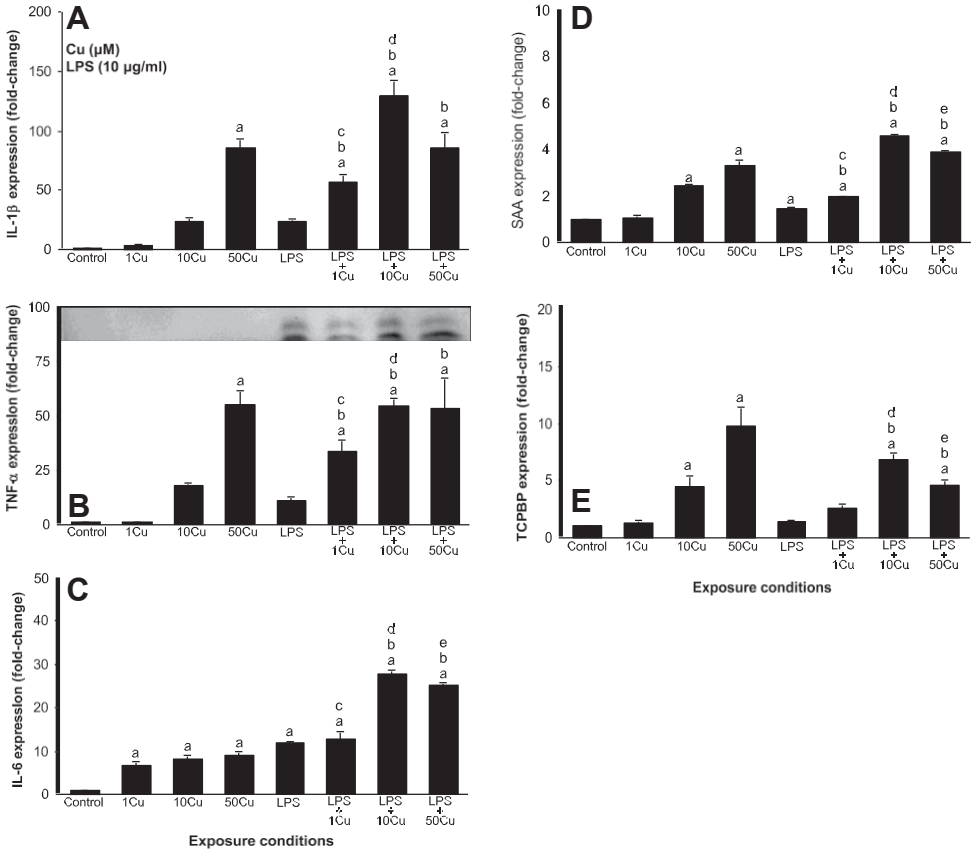

Fig. 2. Gene expression of interleukin-1 b (IL-1b) (A), tumour necrosis factor-a (TNFa) (B), interleukin-6 (IL-6) (C), serum amyloid A (SAA) (D) and trout C-polysaccharide binding protein (TCPBP) (E) in rainbow trout macrophages exposed to copper (1, 10 or 50 mM) or/and LPS (10 mg/ml) for 24 h. Gene expression was analyzed by real-time PCR and normalized to 18S rRNA. Values represent the means and S.E. (n ¼ 4). Significant differences are: a vs. control; b vs. LPS; c vs. 1 mM copper; d vs. 10 mM copper; e vs. 50 mM copper ($P < 0.05$). TNFa secretion to the supernatant was measured and a representative western blot was included in the Fig. 2B (n ¼ 4).

HKM culture supernatants. No differences were detected between the concentration of TNFa detected in the supernatant of cells exposed to LPS and those exposed to the mixture of LPS plus copper (Fig. 2B).

Regarding APP genes, the concentrations of 10 and 50 mM copper induced a significant increase in the expression of SAA and TCPBP genes (Fig. 2D and E). Dose-dependence was clear for both genes where the effect was more evident for TCPBP (4 and 10-fold increase after incubation with 10 and 50 mM copper, respectively). Exposure of rainbow trout HKM to LPS alone induced a significant increase only in SAA mRNA abundance whereas TCPBP was unaltered.

LPS plus all concentrations of copper induced an increase in SAA mRNAs, when compared to control or LPS. Furthermore, all conditions caused an increase higher than the respective concentration of copper alone. For SAA gene expression, some interactions occurred between LPS and copper, namely a potentiation for LPS plus 1 mM copper and a synergism for LPS plus 10 and 50 mM copper. LPS plus 10 and 50 mM copper caused an increase in TCPBP transcription, when compared to control to LPS or to the same concentrations of copper alone. A potentiation occurred for LPS plus 10 mM copper, while for LPS plus 50 mM antagonism was observed.

Exposure of HKM to all copper concentrations caused a dose dependent increase in NADPH oxidase and GPx gene expression when compared to control values (Fig. 3). NADPH oxidase increased 7, 12 and 28-fold after incubation with 1, 10 and 50 mM copper, respectively (Fig. 3A); GPx increased 4, 5 and 7-fold after incubation with 1, 10 and 50 mM copper, respectively (Fig. 3B). LPS alone also caused a significant increase in both mRNAs. LPS plus 1, 10 and 50 mM copper induced an increase in NADPH oxidase and GPx transcription when compared to control; however, this increase was significantly lower when compared to LPS alone. Furthermore, LPS was observed as antagonistic in respect to copper induced increases in NADPH oxidase (plus 10 or 50 mM copper) and GPx (1 and 10 mM copper) mRNA abundance.

The expression of HMGB1 was not altered (Fig. 4A), whereas, an increase in caspase 6 mRNA abundance was induced by all treatments compared to control. LPS plus 10 mM copper induced a significant increase compared to LPS. Furthermore, LPS plus 1 mM and LPS plus 10 mM copper caused a significantly higher increase than 1 or 10 mM copper alone, respectively, suggesting an additive effect (Fig. 4B).

4. Discussion

The present study concerns the in vitro effects of copper and LPS on the expression of genes related with immune function, oxidative stress status and cell death, using rainbow trout HKM as a model. Copper effects are evaluated both alone and combined with LPS, since fish in aquatic environment can be simultaneously exposed to contaminants and pathogens.

The concentrations of copper tested on the cell viability assay were based on previous in vitro studies conducted in rainbow trout head kidney cells [21]. The copper concentrations selected for the main experiment (1, 10 and 50 mM) took into account the cell viability results and the knowledge that fish captured at impacted sites had concentrations in plasma that reach 50 mM [22].

The present results set the LC50-24 h for copper in rainbow trout HKM at 60 mM, which is comparable to those obtained with

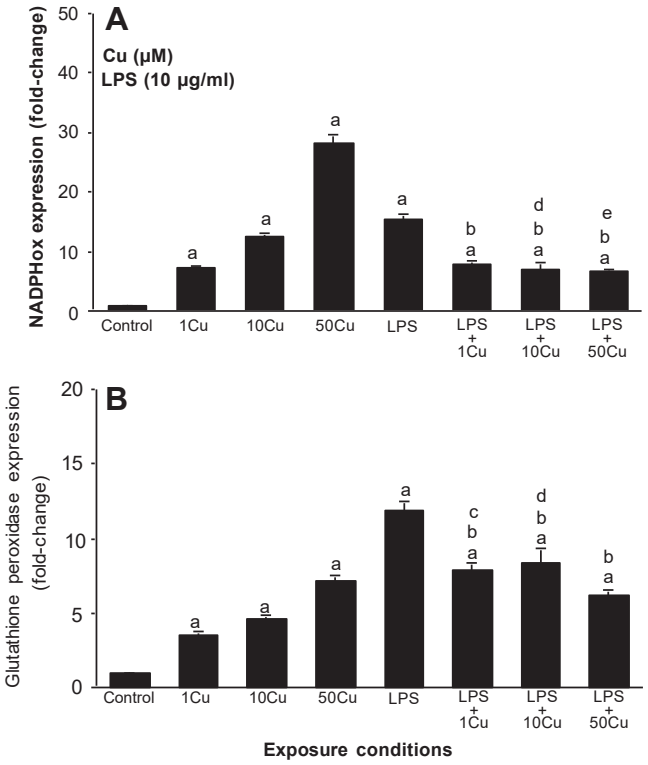

Fig. 3. Gene expression of NADPH oxidase (A) and glutathione peroxidase (B) in rainbow trout macrophages exposed to copper (1, 10 or 50 mM) or/and LPS (10 mg/ml) for 24 h. Gene expression was analyzed by real-time PCR and normalized to 18S rRNA. Values represent the means and S.E. (n ¼ 4). Significant differences are: a vs. control; b vs. LPS; c vs. 1 mM copper; d vs. 10 mM copper; e vs. 50 mM copper ($P < 0.05$).

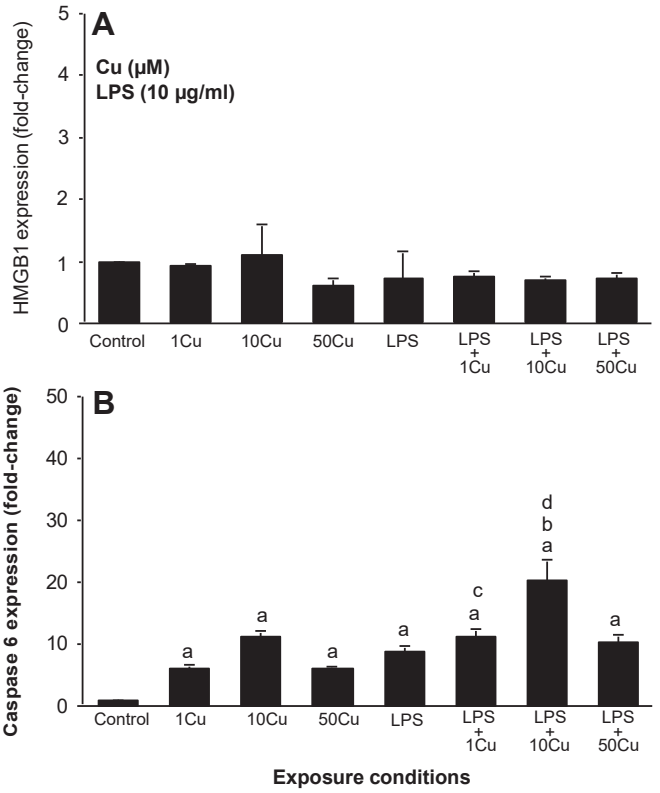

Fig. 4. Gene expression of HMGB1 (A) and caspase 6 (B) in rainbow trout macrophages exposed to copper (1, 10 or 50 mM) or/and LPS (10 mg/ml) for 24 h. Gene expression was analyzed by real-time PCR and normalized to 18S rRNA. Values represent the means and S.E. (n ¼ 4). Significant differences are: a vs. control; b vs. LPS; c vs. 1 mM copper; d vs. 10 mM copper ($P < 0.05$).

a grass carp kidney cell line exposed to copper (50% viability at 70 mM) [23]. However, rainbow trout HKM showed higher resistance to copper considering that 100% mortality was reached at around 2000 mM, while for carp cell line it was reached at a concentration around 400 mM.

Cytokines, such as TNFa, IL-1 and IL-6, have a fundamental role in the regulation of the fish immune response [24]. In the present study, the exposure of rainbow trout HKM to copper, especially at the highest concentration (50 mM), caused a general increase in IL-1b, TNFa and IL-6 mRNA indicating a stimulatory action upon proinflammatory processes. Similarly, other authors obtained an increased transcription of other immune related-genes (e.g. TGF-b) in the whole head kidney of Morone saxatilis [15] and Solea senegalensis [16] exposed to copper in vivo. Previous studies with mammals attributed this action to copper's capacity to activate the transcription factor NF-kappaB [25,26].

The increasing tendency observed in the transcription of IL-1b and TNFa and the significant increase of IL-6 after LPS stimulation was expected and confirms previous studies using rainbow trout HKM under similar experimental conditions [1,7,27]. Copper and LPS interact positively, inducing an increase in cytokine expression in HKM suggesting a possible increase in inflammatory status.

The relation between the secretion of a given cytokine and its mRNA abundance does not always reflect the level of protein. Interestingly, increases in TNFa mRNA expression induced by 50 mM copper failed to induce TNFa protein secretion suggesting that translation of the TNFa mRNA or secretion of the TNFa protein is somehow inhibited. In line with the current results, a few studies available in mammals showed that copper exposure caused an increase in TNFa mRNA transcription but not in TNFa secretion in human peripheral blood monocytes [28]. Furthermore, human THP-1 macrophages incubated for 24 h with copper had unchanged TNFa secretion [29]. On the other hand, copper induced TNFa secretion in human glioblastoma cells [30]. To our knowledge, this is the first study identifying post-transcriptional effects of copper on TNFa expression and secretion in fish.

In the present study, 10 and 50 mM copper caused an increase in SAA and TCPBP mRNA expression. Previously, it was observed that waterborne cadmium caused an increase in plasmatic C-reactive protein of Catla catla [31], and that copper induced amyloid precursor protein mRNA expression in the brain of mice [32]. Current LPS exposure also induced the expression of SAA, corroborating the findings of Goetz et al. (2004) who detected an upregulation of SAA mRNA transcription in rainbow trout HKM stimulated during 12 h with 10 mg/ml of LPS. Furthermore, rainbow trout exposed in vivo to LPS had increased levels of SAA mRNA transcripts in head kidney as do isolated head kidney leukocytes incubated for 1 h with LPS (25 mg/ml) [6]. Contrastingly, current LPS exposure did not induce the transcription of TCPBP in HKM, which is in agreement with previous findings where rainbow trout challenged in vivo with an inflammatory stimulus (bacteria) had unaltered TCPBP mRNA transcripts in the liver [33].

In mammals, SAA gene expression is regulated by inflammatory cytokines, such as IL-1b, TNFa and IL-6 [3]. In fish, a correlation was observed between increased production of SAA and elevated levels of cytokine mRNA either in hepatocytes or extra-hepatic cells [5,34]. In the present study, an association between IL-1b or IL-6 and SAA expression is apparent suggesting its involvement in the APP response. For TCPBP, LPS alone had no effect although LPS and 10 mM copper interacted positively (synergism), while LPS plus 50 mM copper induced antagonism, revealing a concentration-dependent

response to copper. Although hepatocytes are the most important site of APP production, the current results demonstrated SAA and TCPBP expression in fish macrophages, indicating a role of APP and a novel role for TCPBP in the local response to inflammation.

One of the most potent sources of superoxide anions is the phagocyte respiratory burst NADPH oxidase system. Additionally, exposure to environmental contaminants provides an exogenous source of superoxide. To counteract the potential damage caused by oxyradicals, cells have developed a number of protective mechanisms that includes antioxidant enzymes, such as GPx. In the present study, NADPH oxidase and GPx gene expression was increased in rainbow trout HKM after 24 h exposure to copper, LPS and LPS plus copper. These results support the view of copper as an inducer of ROS production in fish [35]. Furthermore, it was previously found that copper induced GPx transcription in gills of brown trout [36] and in the liver of Pomatoschistus microps [37]. The results currently obtained for GPx expression following exposure to LPS is in agreement with Lazado et al. (2010) [38] who reported an increased expression of GPx and catalase in Atlantic cod HKM stimulated in vitro for 24 h with Pseudomonas sp. These results indicate that the antioxidant defences of the cells were activated to counteract the pro-oxidative potential of copper (a redox-active metal), LPS and the mixture of both.

No studies are available concerning the effects of copper concentration on NADPH oxidase, neither in fish nor in mammals. Regarding LPS exposure, and in line with the present findings, Boltaña et al. (2009) [39] obtained an increased expression of NADPH oxidase system mRNAs in rainbow trout HKM stimulated with 10 mg/ml LPS for 12 h. In our study the differences between the effects caused by copper or LPS alone and those obtained after exposure to the mixture, revealed an antagonistic interaction, possibly due to a cumulative effect of both stimulus and subsequent ROS overproduction, impairing cell metabolism.

High mobility group box 1 (HMGB1) can be released by cells in response to inflammatory stimuli [40]. Under the present conditions HMGB1 mRNA abundance was unchanged, suggesting that cells were not undergoing necrosis. However, the exposure to copper, LPS and both potentially results in apoptosis as suggested by enhanced caspase 6 mRNA potentially an early sign of apoptosis. Previous in vitro studies demonstrated that copper induced the activation of caspase 3 suggesting its involvement in copper induced apoptotic death [41]. Additionally, copper induces apoptosis in rainbow trout hepatocytes and cultured gill cells [42,43].

5. Conclusions

In conclusion, present results indicate that all tested genes are induced in HKM by copper in a dose effect manner, except for the cell death HMGB gene. LPS, in general, induced the expression of cytokines. Also acute phase protein genes (SAA) and oxidative stress genes are sensitive to LPS challenge. When both stimuli were applied together, at the concentrations of 1 and 10 uM Cu, synergistic effects were found in cytokine gene response, but not in oxidative stress genes or cell death genes. Therefore, such a higher response of the HKM to concurrent stimuli suggests a high degree of sensitivity of these immune cells in front of different types of challenges.

The studied cytokines may play a role not only in responding to immune challenges, as expected, but also to other challenges affecting the cell integrity such as heavy metal treatments. The increase in TNFa mRNA expression induced by 50 mM copper failed to induce TNFa protein secretion suggesting that translation of the TNFa mRNA is somehow inhibited and that the increase in the expression of a given gene is not always accompanied by the correspondent translation into protein.

Finally, more work has to be done on the mechanisms behind the communication among the regulatory systems of such a network as the alteration of the patterns of gene expression suggests.

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References

[1] Mackenzie S, Planas JV, Goetz FW. LPS-stimulated expression of a tumour necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages. Dev Comp Immunol 2003;27:393e400.
[2] Mackenzie S, Iliev D, Liarte C, Koskinen H, Planas JV, Goetz FW, et al. Transcriptional analysis of LPS-stimulated activation of trout (Oncorhynchus mykiss) monocyte/macrophage cells in primary culture treated with cortisol. Mol Immunol 2006;43:1340e8.
[3] Bayne CJ, Gerwick L, Fujiki K, Nakao M, Yano T. Immune-relevant (including acute phase) genes identified in the livers of rainbow trout, Oncorhynchus mykiss, by means of suppression subtractive hybridization. Dev Comp Immunol 2001;25:205e17.
[4] Thorn CF, Whitehead AS. Differential transcription of the mouse acute phase serum amyloid A genes in response to pro-inflammatory cytokines. Amyloid 2002;9:229e36.
[5] J4rgensen JB, Lunde H, Jensen L, Whitehead AS, Robertsen B. Serum amyloid A transcription in Atlantic salmon (Salmo salar L.) hepatocytes is enhanced by stimulation with macrophage factors, recombinant human IL-1b, IL-6 and TNFa or bacterial lipopolysaccharide. Dev Comp Immunol 2000;24:553e63.
[6] Villarroel F, Casado A, Vásquez J, Matamala E, Araneda B, Amthauer R, et al. Serum amyloid A: a typical acute-phase reactant in rainbow trout? Dev Comp Immunol 2008;32:1160e9.
[7] Goetz FW, Iliev DB, McCauley LAR, Liarte CQ, Tort LB, Planas JV, et al. Analysis of genes isolated from lipopolysaccharide-stimulated rainbow trout (Oncorhynchus mykiss) macrophages. Mol Immunol 2004;41:1199e210.
[8] Ewart KV, Williams J, Richards RC, Gallant JW, Melville K, Douglas SE. The early response of Atlantic salmon (Salmo salar) macrophages exposed in vitro to Aeromonas salmonicida cultured in broth and in fish. Dev Comp Immunol 2008;32:380e90.
[9] Novoa B, Figueras A, Ashton I, Secombes CJ. In vitro studies on the regulation of rainbow trout (Oncorhynchus mykiss) macrophage respiratory burst activity. Dev Comp Immunol 1996;20:207e16.
[10] Tafalla C, Novoa B. Respiratory burst of turbot (Scophthalmus maximus) macrophages in response to experimental infection with viral haemorrhagic septicaemia virus (VHSV). Fish Shellfish Immunol 2001;11:727e34.
[11] Iliev DB, Liarte CQ, Mackenzie S, Goetz FW. Activation of rainbow trout (Oncorhynchus mykiss) mononuclear phagocytes by different pathogen associated molecular pattern (PAMP) bearing agents. Mol Immunol 2005;42:1215e23.
[12] Datta S, Ghosh D, Saha DR, Bhattacharaya S, Mazumder S. Chronic exposure to low concentration of arsenic is immunotoxic to fish: role of head kidney macrophages as biomarkers of arsenic toxicity to Clarias batrachus. Aquat Toxicol 2009;92:86e94.
[13] Tellez-Bañuelos MC, Santerre A, Casas-Solis J, Bravo-Cuellar A, Zaitseva G. Oxidative stress in macrophages from spleen of Nile tilapia (Oreochromis niloticus) exposed to sublethal concentration of endosulfan. Fish Shellfish Immunol 2009;27:105e11.
[14] Handy RD. Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? Comp Biochem Physiol A 2003;135:25e38.
[15] Geist J, Werner I, Eder KJ, Leutenegger CM. Comparisons of tissue-specific transcription of stress response genes with whole animal endpoints of adverse effect in striped bass (Morone saxatilis) following treatment with copper and esfenvalerate. Aquat Toxicol 2007;85:28e39.
[16] Prieto-Álamo MJ, Abril N, Osuna-Jiménez I, Pueyo C. Solea senegalensis genes responding to lipopolysaccharide and copper sulphate challenges: large-scale identification by suppression subtractive hybridization and absolute quantification of transcriptional profiles by real-time RT-PCR. Aquat Toxicol 2009;91:312e9.
[17] Osuna-Jiménez I, Williams TD, Prieto-Álamo MJ, Abril N, Chipman JK, Pueyo C. Immune- and stress-related transcriptomic responses of Solea senegalensis stimulated with lipopolysaccharide and copper sulphate using heterologous cDNA microarrays. Fish Shellfish Immunol 2009;26:699e706.
[18] Manzl C, Krumschnabel G, Schwarzbaum PJ, Dallinger R. Acute toxicity of cadmium and copper in hepatopancreas cells from the Roman snail (Helix pomatia). Comp Biochem Physiol C 2004;138:45e52.

[19] Murphy JK, Hoyal CR, Livingston FR, Forman HJ. Modulation of the alveolar macrophage respiratory burst by hydroperoxides. Free Radic Biol Med 1995;18:37e45.
[20] Kirkham P. Oxidative stress and macrophage function: a failure to resolve the inflammatory response. Biochem Soc Trans 2007;35:284e7.
[21] Gagnon A, Jumarie C, Hontela A. Effects of Cu on plasma cortisol and cortisol secretion by adrenocortical cells of rainbow trout (Oncorhynchus mykiss). Aquat Toxicol 2006;78:59e65.
[22] Kamunde C, MacPhail R. Bioaccumulation and hepatic speciation of copper in rainbow trout (Oncorhynchus mykiss) during chronic waterborne copper exposure. Arch Environ Contam Toxicol 2008;54:493e503.
[23] Tan F, Wang M, Wang W, Lu Y. Comparative evaluation of the cytotoxicity sensitivity of six fish cell lines to four heavy metals in vitro. Toxicol In Vitro 2008;22:164e70.
[24] Engelsma M, Huising M, Muiswinkel W, Flik G, Kwang J, Savelkoul H, et al. Regulation of interleukin 1 beta RNA expression in the common carp, Cyprinus carpio L. Vet Immunol Immunopathol 2002;87:467e79.
[25] Kennedy T, Ghio AJ, Reed W, Samet J, Zagorski J, Quay J, et al. Copper-dependent inflammation and nuclear factor-kappaB activation by particulate air pollution. Am J Respir Cell Mol Biol 1998;19:366e78.
[26] Persichini T, Percario Z, Mazzon E, Colasanti M, Cuzzocrea S, Musci G. Copper activates the NF-kappaB pathway in vivo. Antioxid Redox Signal 2006;8:1897e904.
[27] Roher N, Callol A, Planas JV, Goetz FW, Mackenzie SA. Endotoxin recognition in fish results in inflammatory cytokine secretion not gene expression. Innate Immun 2010 (in press).
[28] Scuderi P. Differential effects of copper and zinc on human peripheral blood monocyte cytokine secretion. Cell Immunol 1990;126:391e405.
[29] Wataha JC, Ratanasathien S, Hanks CT, Sun Z. In vitro IL-1 beta and TNF-alpha release from THP-1 monocytes in response to metal ions. Dent Mater 1996;12:322e7.
[30] Becaria A, Bondy SC, Campbell A. Aluminum and copper interact in the promotion of oxidative but not inflammatory events: implications for Alzheimer's disease. J Alzheimer Dis 2003;5:31e8.
[31] Paul I, Mandal C, Allen AK, Mandal C. Glycosylated molecular variants of C-reactive proteins from the major carp Catla catla in fresh and polluted aquatic environments. Glycoconj J 2001;18:547e56.
[32] Lu J, Zheng YL, Wu DM, Sun DX, Shan Q, Fan SH. Trace amounts of copper induce neurotoxicity in the cholesterol-fed mice through apoptosis. FEBS Lett 2006;580:6730e40.
[33] Raida MK, Buchmann K. Innate immune response in rainbow trout (Oncorhynchus mykiss) against primary and secondary infections with Yersinia ruckeri O1. Dev Comp Immunol 2009;33:35e45.
[34] Gonzalez SF, Buchmann K, Nielsen ME. Ichthyophthirius multifiliis infection induces massive up-regulation of serum amyloid A in carp (Cyprinus carpio). Vet Immunol Immunopathol 2007;115:172e8.
[35] Bopp SK, Abicht HK, Knauer K. Copper-induced oxidative stress in rainbow trout gill cells. Aquat Toxicol 2008;86:197e204.
[36] Hansen BH, Rømma S, Garmo ØA, Pedersen SA, Olsvik PA, Andersen RA. Induction and activity of oxidative stress-related proteins during waterborne Cd/Zn-exposure in brown trout (Salmo trutta). Chemosphere 2007;67:2241e9.
[37] Vieira LR, Gravato C, Soares AM, Morgado F, Guilhermino L. Acute effects of copper and mercury on the estuarine fish Pomatoschistus microps: linking biomarkers to behaviour. Chemosphere 2009;76:1416e27.
[38] Lazado CC, Caipang CMA, Gallage S, Brinchmann MF, Kiron V. Expression profiles of genes associated with immune response and oxidative stress in Atlantic cod, Gadus morhua head kidney leukocytes modulated by live and heat-inactivated intestinal bacteria. Comp Biochem Physiol B 2010;155:249e55.
[39] Boltaña S, Doñate C, Goetz FW, MacKenzie S, Balasch JC. Characterization and expression of NADPH oxidase in LPS-, poly(I: C)- and zymosan-stimulated trout (Oncorhynchus mykiss W.) macrophages. Fish Shellfish Immunol 2009;26:651e61.
[40] Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 2007;81:1e5.
[41] Nawaz M, Manzl C, Lacher V, Krumschnabel G. Copper-induced stimulation of extracellular signal-regulated kinase in trout hepatocytes: the role of reactive oxygen species, $Ca^{2Þ}$, and cell energetics and the impact of extracellular signal-regulated kinase signaling on apoptosis and necrosis. Toxicol Sci 2006;92:464e75.
[42] Krumschnabel G, Manzl C, Berger C, Hofer B. Oxidative stress, mitochondrial permeability transition, and cell death in Cu-exposed trout hepatocytes. Toxicol Appl Pharmacol 2005;209:62e73.
[43] Mazon AF, Nolan DT, Lock RA, Fernandes MN, Wendelaar Bonga SE. A short-term in vitro gill culture system to study the effects of toxic (copper) and non-toxic (cortisol) stressors on the rainbow trout, Oncorhynchus mykiss (Walbaum). Toxicol In Vitro 2004;18:691e701.