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Immunostimulatory effects of prolactin on TLR1 and TLR5M in SHK-1 cells infected with *Piscirickettsia salmonis*

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ABSTRACT: The innate immune system is the first line of defense against infection by pathogens. It consists of various elements, including Toll-like receptors (TLRs), which recognize molecular patterns associated with pathogens and trigger the immune response, through activation of important transcription factors such as NF-κB, which are usually found sequestered in the cytoplasm by IκBα until it receives the release signal. *Piscirickettsia salmonis* causes piscirickettsiosis or salmonid rickettsial septicemia, a disease of great importance in Chile, representing 79.4% of the secondary mortality in important species such as *Salmo salar*, which is reflected in the Chilean economy. Prolactin (PRL) is a peptide hormone which has immunomodulating functions in mammals and some fish. Olavarría et al. (2010, *J Immunol 185:3873–3883*) determined its ability to increase the respiratory burst, its relationship with the JAK/STAT pathway, and the expression of interleukin IL-1β in *Sparus aurata*. Therefore, the present study was intended to establish a possible correlation and modulation between the signal transduction pathway of PRL (JAK/STAT), the pathways of NF-κB, and TLRs, in an infection caused by *P. salmonis* in salmon head kidney (SHK-1) cells of *S. salar*. Stimulus with native PRL from *S. salar* was performed, and gene expression was analyzed for IL-1β, IκBα, TLR1, and TLR5M (membrane-bound form). In addition, the effect of PRL in the nuclear translocation of the transcription factor NF-κB and the possible involvement of JAK2 were analyzed by using a pharmacological inhibitor of this kinase. The results show a positive modulation of PRL in all analyzed genes and a significant increase in the translocation of NF-κB, recording a maximum at 2 h post-treatment, supporting the stimulatory hypothesis of PRL.

KEY WORDS: Piscirickettsiosis · Salmonid rickettsial septicemia · *Salmo salar* · Toll-like receptors · Prolactin · Immunostimulants

INTRODUCTION

Constant economic losses in the salmon industry due to diverse pathogens have sparked a search for new molecules to mitigate the situation. Prolactin (PRL) is one such molecule due to its immunomodulating functions (Bole-Feysot et al. 1998). Prolactin is a hormone produced in the pituitary gland (Sodhi & Tripathi 2008) and belongs to the class 1 cytokine family, a group comprised of proteins such as erythropoietin and growth hormone (Bazan 1989). Over 300 functions have been attributed to PRL to date, and have been classified into the following 6 groups: water and electrolyte balance, growth and development, endocrinology, metabolism, reproduction, and immunoregulation (Bole-Feysot et al. 1998, Sakamoto & McCormick 2006). As in mammals, PRL has been shown to have immunoregulatory functions in fish. Stimulation of PRL in fish promotes mitotic activity of leukocytes (Yada et al. 1999), increasing phagocytic activity of macrophages (Sakai et al. 1996) and positively regulating gene expression of proinflammatory cytokine interleukin (IL)-1β (Olavarría et al. 2010). Moreover, in *Sparus aurata* L., PRL in leuko-

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cytes was observed to mediate effects through the Janus kinase/signal transducer and activation of transcription (JAK/STAT) pathway (Olavarría et al. 2010), a phenomenon that has also been observed in mammals (Schindler & Darnell 1995).

However, through use of pharmacological inhibitors and an electromobility shift assay, JAK/STAT and nuclear factor (NF)-κB signaling pathways were found to be activated on *S. aurata* phagocytes stimulated with PRL (Olavarría et al. 2010). NF-κB belongs to a family of inducible transcription factors that participate in immune response through activation by the Toll-like receptors (TLRs; Akira et al. 2006). TLRs are present in cells belonging to the immune system, recognizing conserved motifs, termed pathogen-associated molecular patterns, which are found predominantly in microorganisms, but not in vertebrates. Activation of these receptors triggers immediate defensive responses, including the production of antimicrobial peptides and cytokines (Akira 2003). There is an important difference between the identified TLR5 in fish and mammals: in mammals there exists only the membrane-bound form (TLR5M), but fish possess also a soluble orthologue (TLR5S) (Salazar et al. 2016).

The mechanisms involved in activation of NF-κB and JAK through PRL receptors are not yet completely understood, but current evidence indicates that JAK could be capable of directly mediating phosphorylation of IκBα, a cytoplasmic repressor of NF-κB that disassociates when phosphorylated. Thus, it could permit the translocation of NF-κB to the nucleus and, consequently, the initiation of genetic transcription (Olavarría et al. 2010). This regulation could be possible, given that such a mechanism has been described when explaining the neuroprotective effect mediated by erythropoietin, an NF-κB dependent cytokine belonging to the same family as PRL (Digicaylioglu & Lipton 2001).

Although Chile is among the world's leading salmon producers, this industry is widely affected by diverse pathogens, some of which are devastating for production. Piscirickettsiosis is one such disease, causing losses during the fattening stage of the 3 most important salmonids of the Chilean aquaculture industry. During the infectious cycle, a series of factors coexist, including the traits of pathogen and fish, and the cultivation conditions to which the fish are subjected. *Piscirickettsia salmonis* is the causal agent of piscirickettsiosis, or salmonid rickettsial septicemia (SRS), and this pathogen is responsible for approximately US$ 100 million in annual losses (McCarthy et al. 2008). *P. salmonis* was the first rickettsia to be isolated and characterized in fish, initially being identified in Chile by Bravo & Campos (1989), and subsequently characterized by Fryer et al. (1990) and Cvitanich et al. (1991). SRS was first described in coho salmon *Oncorhynchus kisutch*, but quickly spread to other salmonid hatcheries in Chile, and has caused up to 90% mortality in some aquaculture centers.

P. salmonis is a Gram-negative, non-motile, aerobic, facultative intracellular bacterium that is predominantly coccoid (measuring between 0.5 and 1.5 µm), affecting cultivated fish in both estuaries and the sea (Fryer & Hedrick 2003). This bacterium has been described in distinct salmonid species such as Coho salmon, Atlantic salmon *Salmo salar*, and rainbow trout *O. mykiss* (Toranzo et al. 2005). Classic clinical signs of infection include erratic swimming, slow movement when near the water surface, punctiform ulcers, and white nodules on the skin. Such symptoms are in addition to livers marked by white nodules and petechial hemorrhages, principally in the pyloric caeca (Cvitanich et al. 1991, Rozas & Enriquez 2014).

Salazar et al. (2016) used time-course analyses of *P. salmonis* infection in the anterior kidney cell line of *S. salar* (SHK-1) and revealed that the bacterium was able to modulate the expression of the studied receptors, i.e. TLRs, in addition to Myd88 and IL-1β, 2 important molecules in the TLR signaling chain. These observations could indicate a way in which this bacterium evades the host's immune response and causes high mortality rates of Atlantic salmon in the Chilean aquaculture industry (Isla et al. 2014). Taking this background into consideration, here we aimed to evaluate whether PRL has an immunostimulatory effect on SHK-1 cells infected *in vitro* with *P. salmonis* through infection kinetics expression of TLR1 and TLR5M, IL-1β and IκBα, together with the evaluation of the effect of a pharmacological inactivation of JAK2 and the translocation of NF-κB in a kinetic stimulation with PRL.

MATERIALS AND METHODS

The *Piscirickettsia salmonis* LF-89 (ATCC VR-1361) strain was grown in tryptic soy broth (Merck), supplemented according to Vera et al. (2012).

Collection of pituitary glands and prolactin extraction

Pituitary glands used for the purification of prolactin were obtained from heads of adult male and

female Atlantic salmon (≥2 kg) from the Marine-Harvest primary production center in Puerto Montt, Chile, following standard procedures of the company. Pituitary glands were quickly extracted, placed in liquid nitrogen, and kept at −80°C until analysis. Extraction of PRL was performed according to protocols outlined by Andersen et al. (1989) and used by Paredes et al. (2013). The quality of purified PRL was confirmed through Western blot analysis and high-pressure liquid chromatography. All conditions used 250 ng ml^{-1} of PRL, experimentally determined in SHK-1 cells, and were followed by an analysis of IL-1β expression (Fig. S1 in the Supplement, available at www.int-res.com/articles/suppl/d118p237_supp.pdf).

Cell cultivation and treatments

Salmon head kidney (SHK-1) cells (catalogue no. 97111106, Health Protection Agency, Culture Collection; www.pheculturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=97111106&collection=ecacc_gc), were maintained in an L-15 Leibovitz medium (Hyclone) supplemented with 5% fetal bovine serum (FBS) (Invitrogen). These cells were stimulated for 2, 4, and 16 h with 500 ng ml^{-1} of purified PRL (we standardized PRL concentration prior to use by stimulating SHK-1 cells with concentrations of 50 to 1000 ng ml^{-1}, finding the optimum to be 250 ng ml^{-1}; see Fig. S1 in the Supplement. *P. salmonis* was used in a proportion of 100 bacteria per SHK-1 cell. Additionally, 50 µg ml^{-1} of *Vibrio anguillarum* DNA (strain ATCC19264) was used via phenol-chloroform extraction as a positive stimulation control. For some experiments, SHK-1 cells were pretreated for 2 h with 50 to 200 µM of AG-490, a pharmacological inhibitor of JAK2. Adding cold phosphate-buffered saline (PBS) 1× stopped stimulation. All incubations of SHK-1 cells at different times and treatments were performed in triplicate. The cells were then lysed for subsequent total RNA extraction.

Gene expression analysis

RNA was extracted from lysed cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was then treated with 1 U $µg^{-1}$ of DNase I, Amplification Grade (Invitrogen). For reverse transcription, 1 µg of total RNA was used as a template at 42°C for 1 h using M-MLV Reverse Transcriptase (Promega) with oligo-dT18. Quantitative PCR analysis was run with an MXPro 3005x instrument, using GoTaq®qPCR Master Mix (Promega) according to the manufacturer's instructions. The reaction solution was incubated for 5 s at 25°C and for 10 min at 95°C; followed by 40 cycles of 15 s at 95°C, 15 s at 58–62°C, and 15 s at 72°C; with a final cycle of 10 s at 95°C, 10 s at 25°C, 1 s at 70°C, and 1 s at 95°C. For each template, gene expression was normalized with β-actin using the comparative Ct method ($2^{-\Delta\Delta Ct}$). The primers are shown in Table S1 in the Supplement. For each gene and time point (2, 4, and 16 h) analyzed, the PCR reaction was performed in triplicate, using 3 distinct samples.

Immunocytochemistry

Each well was seeded with 3×10^4 SHK-1 cells at 17°C and placed on cultivation slides containing L-15 medium supplemented with 5% FBS. After 48 h, cultivations were incubated with PRL for 15 min, 45 min, 1 h, 2 h, and 6 h. To evaluate the effect of JAK2 on translocation of NF-κB, treatments with AG-490 were carried out prior to stimulation with PRL. As a control, an anti-PRL antibody was used as a pre-treatment at a dilution of 1:200 (Olavarría et al. 2010). In all cases, adding cold PBS stopped stimulation. To reveal the reaction, cultivations were incubated overnight with anti-NF-κB (Santa Cruz Biotechnology) at a dilution of 1:500, which was followed by incubation for 1 h with a second fluorescent antibody marked with Alexa Fluor 488 (Invitrogen) at a dilution of 1:200. The reaction was visualized and photographed using a Zeiss Lab.A1 microscope.

Statistical analysis

All data are represented as the mean ± SE. Differences were analyzed using the Student test, and results were considered significant at $p < 0.05$.

RESULTS

Modulation of TLR1, TLR5M, IκBα, and IL-1β

Samples were taken at different time-points (2, 4, and 16 h) in order to evaluate expression of TLR1 and TLR5M, in addition to IκBα and IL-1β, when infected with *Piscirickettsia salmonis*, and with or without simultaneous stimulation of 250 ng ml^{-1} of PRL, and with *P. salmonis* only as a control. Genetic expression

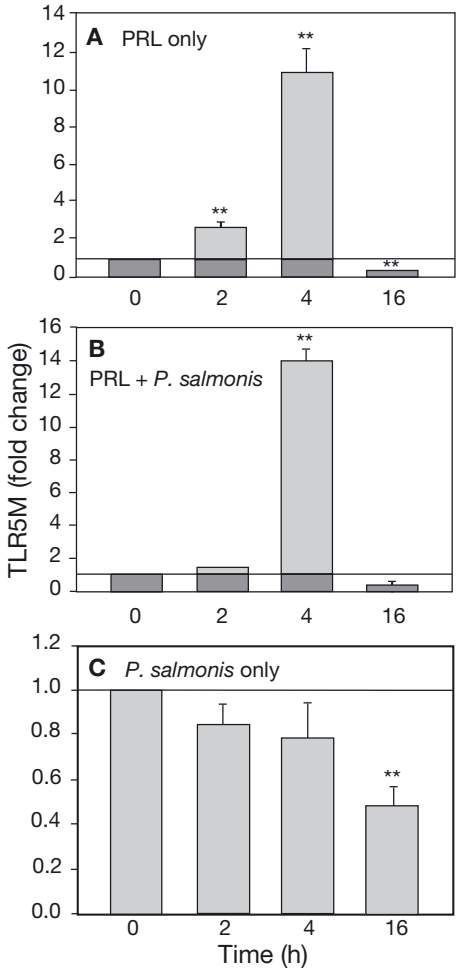

Fig. 1. Time-course expression of TLR5M in (A) cells stimulated with 250 ng ml^{-1} of prolactin (PRL), (B) cells simultaneously incubated with PRL and *Piscirickettsia salmonis* and (C) cells incubated with *P. salmonis* only (control). Each sampling point represents the mean ± SE of 3 samples, in triplicate, and is compared to its corresponding 0 h timepoint (black line; significant differences marked, $^{**}p < 0.001$)

of these samples was measured as the quantity of detectible transcripts through RT-qPCR analysis. First, transcripts of the 2 TLRs were analyzed, then IκBα (a repressor of NF-κB), and, finally, IL-1β, a pro-inflammatory effector cytokine.

Transcripts for the TLR5M gene were observed 4 h post-stimulus (hps) for the PRL-only treatment group, reaching significantly increased transcript levels when compared to the control group (~11-fold) before drastically decreasing over the remainder of the experiment (Fig. 1A). When treating cells with PRL and *P. salmonis*, nearly the same results as those found with the PRL-only treatment were obtained, with gene expression close to 14-fold more than in the control group (Fig. 1B). Control cells were treated with *P. salmonis* alone (Fig. 1C), indicating that the bacterium by itself did not cause an increase in the expression of TLR5M at any time during the analysis. Moreover, it confirmed that PRL was capable of stimulating the expression of TLR5M, both in the presence and absence of the bacterium, at early stages of infection (4 hps); at longer times (16 hps), a drastic decrease in expression of TLR5M was observed in all treatments (Fig. 1). These observations indicated that the effects of the experimental conditions were neither additive nor synergistic.

In the case of TLR1 (4 hps with PRL) a significantly increased expression was observed (~12-fold more than the control). While this expression diminished over time, it remained significantly more expressed than the control group until the end of the experiment (Fig. 2A). Likewise, combined treatment with PRL and *P. salmonis* obtained the same results as stimulation with only PRL, once again indicating a non-synergistic effect (Fig. 2B). The control experiment with *P. salmonis* alone (Fig. 2C) shows no evidence of the ability of the bacterium to significantly affect the expression of TLR1 at all 3 analysis times.

In quantifying transcripts of the gene coding for IκBα, a significant increase in transcripts was observed 2 hps with PRL, reaching approximately 9-fold more than the unstimulated control group. Expression decreased over time, but remained significant until 4 hps (Fig. 3A). In the treatment with PRL and *P. salmonis*, maximum expression was obtained 2 hps and was notably higher than in the treatment with only PRL, reaching approximately 25-fold more than the control group. Increased expression decreased slightly over time, but remained significant until 4 hps (Fig. 3B). The results clearly show a synergistic effect between PRL and the bacterium, which together stimulate an expression 3 times greater than stimulation with PRL alone. However, when administering stimulation 2 h post-infection, notable increases in transcripts were observed at 2 and 4 h, reaching over 100-fold more than values obtained for the control group under all experimental conditions (Fig. 3C). Treatment with *P. salmonis* only (Fig. 3D) generates expression changes in IκBα, but at levels well below those of treatments with PRL alone, or with combined PRL and *P. salmonis*.

Finally, the IL-1β gene showed significantly increased transcripts, detectable through RT-qPCR at 2 hps with PRL only, and reaching approximately 11-fold more than the control group. This expression gradually decreased over the experimental time period, but remained statistically significant when compared to the control group (Fig. 4A). When

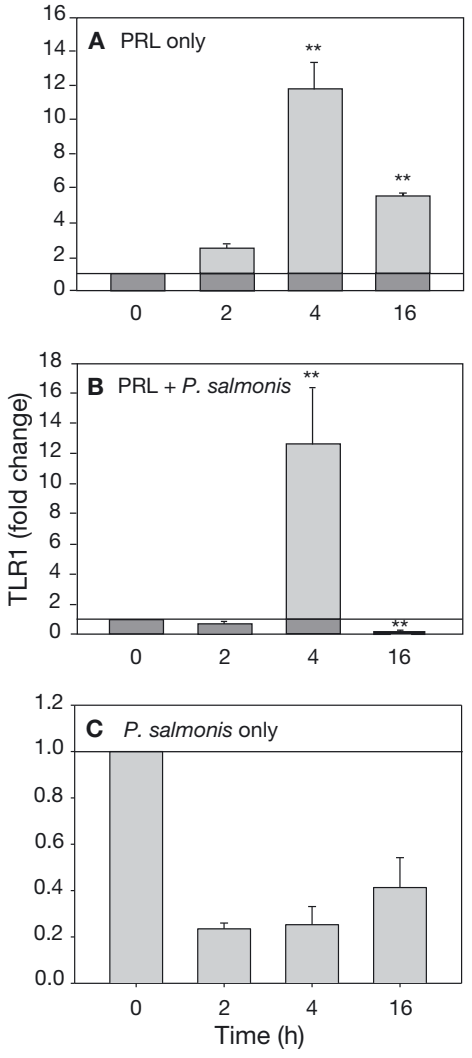

Fig. 2. Time-course expression of TLR1 in (A) cells stimulated with 250 ng ml^{-1} of prolactin (PRL), (B) cells simultaneously incubated with PRL and *Piscirickettsia salmonis* and (C) cells incubated with *P. salmonis* only. Each sampling point was compared to its corresponding 0 h timepoint. Other details as in Fig. 1

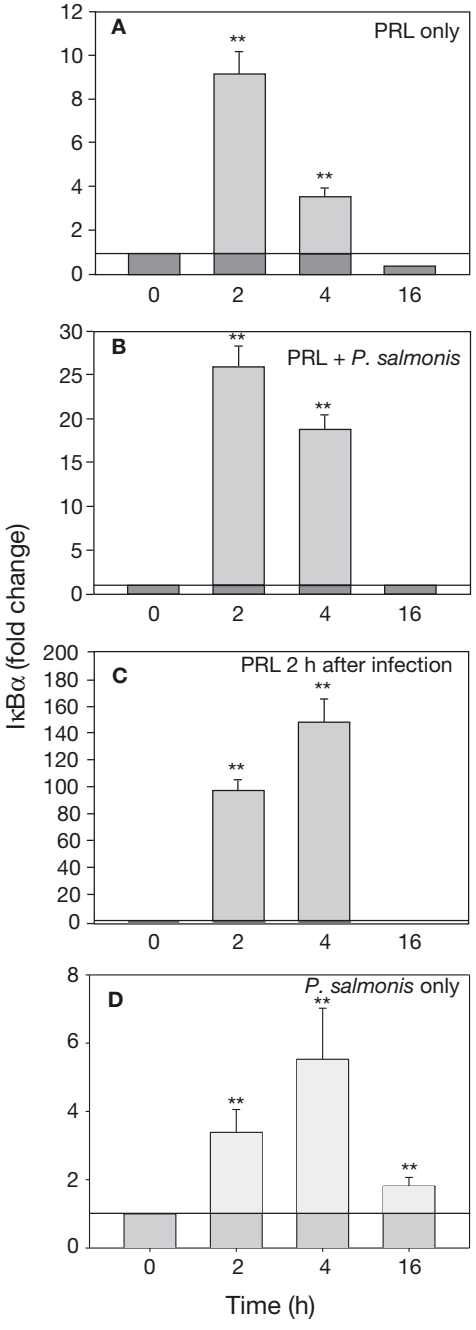

Fig. 3. Time-course expression of IκBα in (A) cells stimulated with 250 ng ml^{-1} of prolactin (PRL), (B) cells simultaneously incubated with PRL and *Piscirickettsia salmonis*, (C) cells treated with PRL 2 h post-infection with *P. salmonis* and (D) cells incubated with *P. salmonis* only. Each sampling point was compared to its corresponding 0 h timepoint. Other details as in Fig. 1

observing cells treated with combined PRL and *P. salmonis*, relatively greater transcript levels were obtained, close to 16-fold more than the control group and higher than levels observed for the PRL-only treatment (Fig. 4B), revealing a possible synergistic effect in the stimulation. When analyzing groups that received stimulus 2 h post-infection, significant increases in transcripts were first obtained at 2 h, but maximum expression was observed at 4 h, in a manner similar to that observed for the analysis of IκBα. The increased expression was 30-fold more than the control group, being the most notable increase obtained for this interleukin (Fig. 4C). In the experiment for cells infected with *P. salmonis* alone (Fig. 4D), a slight increase in the amount of transcript was evident only at 4 h, which was significant with respect to the uninfected control, but much lower than that obtained by stimulation with PRL alone, or combined PRL and *P. salmonis*.

Pre-treatment with AG-490 and a diminished expression of IL-1β in SHK-1 cells

Results obtained for pre-treatment with AG-490 indicated that native PRL purified from the pituitary gland of Atlantic salmon was capable of inducing IL-1β synthesis. Similarly, previously obtained results in *Sparus aurata* indicated that PRL induced the translocation of NF-κB to the nucleus, and additionally, that this translocation could be drastically diminished when cells were treated previously with the inhibitor AG-490. Taking these results into account, RT-qPCR was used to evaluate the effect of JAK2 pharmacological inhibitors on SHK-1 cells stimulated with PRL. Assays were performed using inhibitor concentrations of 50, 100, and 200 µM and a pre-treatment of 2 h. Subsequently, 250 ng ml^{-1} of PRL were added and cells were incubated for 16 h. Analysis revealed a significant decrease of IL-1βtranscript levels (~9-fold less than the control) when cells were treated with 50 to 100 µM of AG-490 (Fig. 5).

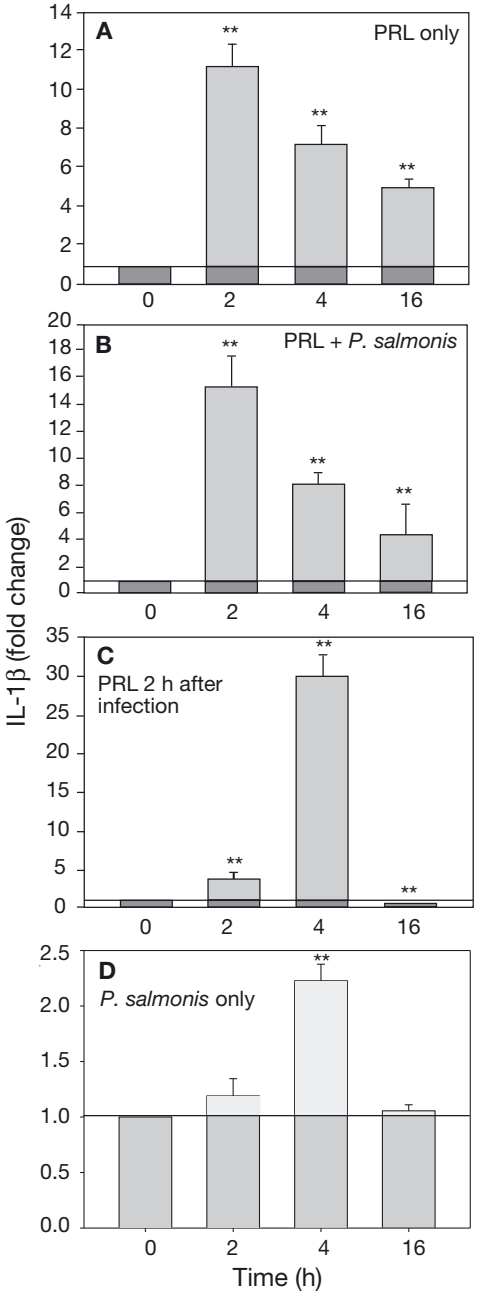

Fig. 4. Time-course expression of IL-1β in (A) cells stimulated with 250 ng ml^{-1} of prolactin (PRL), (B) cells simultaneously incubated with PRL and *Piscirickettsia salmonis*, (C) cells treated with PRL 2 h post-infection with *P. salmonis* and (D) cells incubated with *P. salmonis* only. Each sampling point was compared to its corresponding 0 h timepoint. Other details as in Fig. 1

Translocation of NF-κB to the cellular nucleus

Once a monolayer of SHK-1 cells was grown on culture slides, time-lapse stimulation with 250 ng ml^{-1} of PRL took place for 15, 30, and 45 min, and for 1 and 2 h. We then stopped the reaction and used indirect immunohistochemical detection of NF-κB with Alexa Fluor 488. One of the analyzed wells was pretreated with AG-490 for 2 h. We repeated this procedure 3 times using different samples. As a control, we administered a 4 h pre-treatment with anti-PRL antibody for salmon at a dilution of 1:200. Additionally, a control using only the vehicle (PBS 1×) was used (data not shown). In the quadrant labeled 'with-

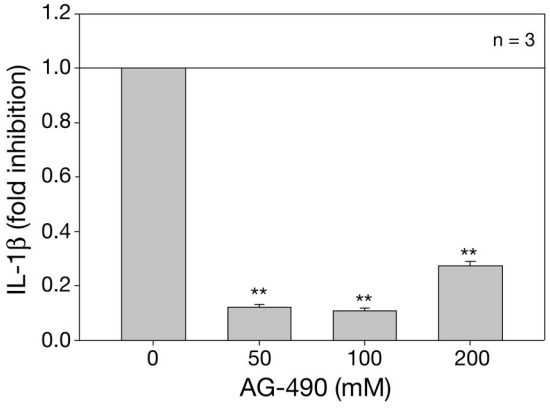

Fig. 5. Relative decreased expression of IL-1β in cells treated with AG-490. SHK-1 cells were stimulated for 16 h with 250 ng ml^{-1} of prolactin (PRL) before treatment for 2 h with AG-490 at the concentrations shown. Transcript levels of proinflammatory cytokine IL-1β were determined through RT-PCR. Genetic expression was normalized with β-actin and is shown relative to the average obtained for unstimulated, control cells. The black line represents the control group over time. Each bar represents the mean ± SD of 3 samples, in triplicate

out PRL,' the NF-κB appeared localized mainly in the cytoplasm, as was expected for unstimulated cells. However, in time-course images, a gradual increase in translocation of NF-κB to the nucleus can be observed, resulting in a massive translocation from 45 min to 2 hps. When cells were treated with AG-490, localization of NF-κB was predominantly cytoplasmic, thus shedding light on the PRL signaling pathway and this transcription factor (Fig. 6).

DISCUSSION

PRL is a very versatile cytokine that has diverse functions in the immune systems of fish (Balm 1997, Harris & Bird 2000, Olavarría et al. 2010, Paredes et al. 2013). Because of the immunomodulating effect of PRL, the aim of this study was to determine its effects on SHK-1 cells uninfected and infected with *Piscirickettsia salmonis*. Salazar et al. (2016) found positive expression of Toll-like receptors TLR1, TLR22, TLR5S, and TLR5M in SHK-1 cells infected with *P. salmonis*. Peña et al. (in press) also demonstrated the likely immunomodulatory role of PRL in the primary culture of leukocytes from the anterior kidney of rainbow trout, in the expression of several TLRs analyzed. The effect is in addition to the described capacities of PRL to stimulate the expression of IL-1β in leukocytes of *Sparus aurata*, and activate the translocation of NF-κB to the nucleus (Olavarría et al. 2010). For all analyses carried out in the present study, native PRL purified from the pituitary gland of *Salmo salar* was used as outlined by established protocols (Andersen et al. 1989).

In analyzing the TLR5 membrane gene (Fig. 1), the fact that *P. salmonis* possesses some types of flagellin on its membrane was taken into consideration (Wilhelm et al. 2006). These flagellins can be recognized by both soluble and membrane TLR5 (Tsujita et al. 2004). Over the experimental period, transcripts for

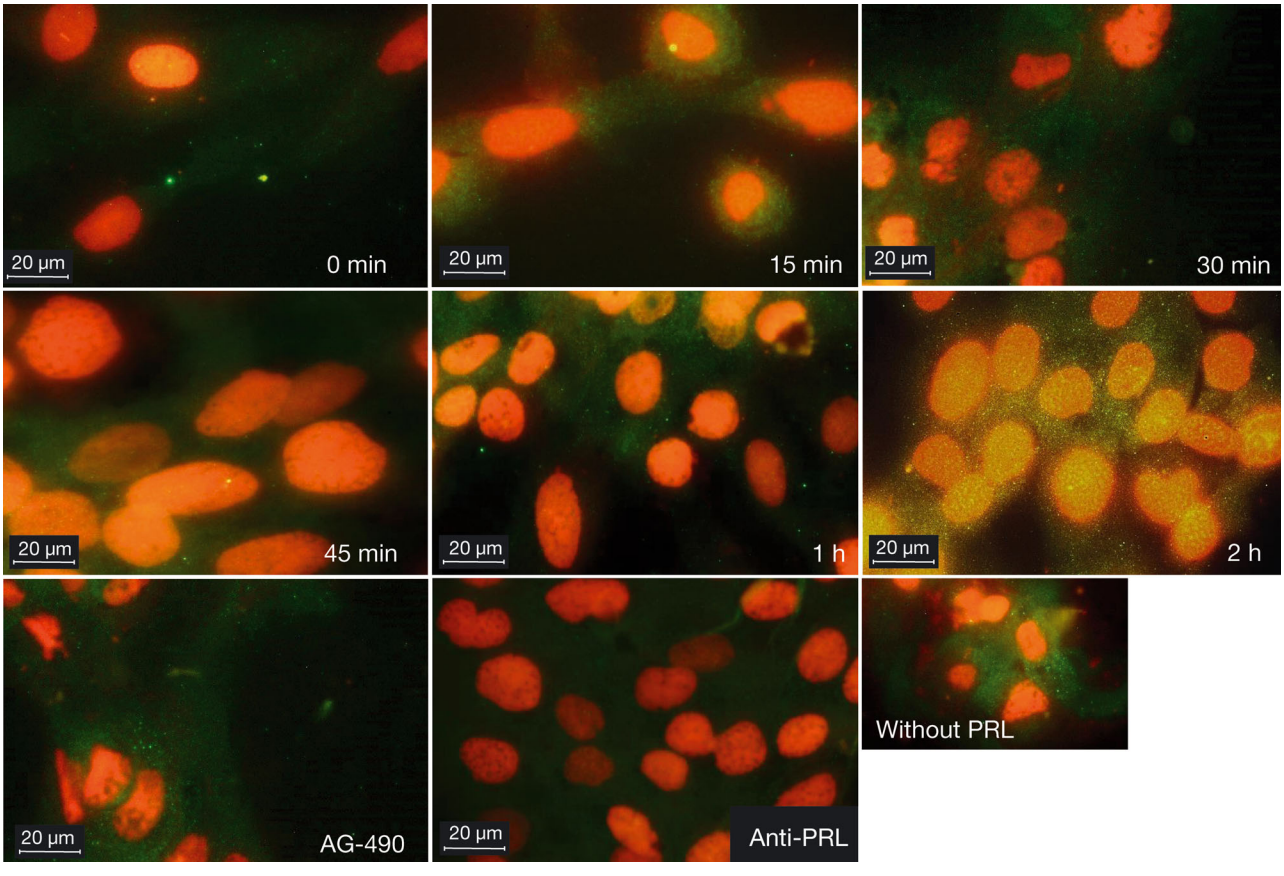

Fig. 6. Time-course translocation of NF-κB transcription factor. SHK-1 cells were stimulated in L-15 medium with 5% bovine serum at 17°C, with 250 ng ml^{-1} purified prolactin (PRL) at the times indicated in each frame. Translocation of NF-κB from cytoplasm to the nucleus was evaluated with anti-NF antibody at a concentration of 1:500, using Alexa 488 fluorescent marker (green) at a dilution of 1:1000. The SHK-1 cell nuclei were stained with propidium iodide (red). Photographs were taken with the same instrument, and the same magnification was used for all frames

this gene evidenced a clear immunostimulation by PRL, reaching 10-fold more than the control 4 hps (Fig. 1A). Moreover, a non-synergistic effect was observed when stimulated and challenged with PRL and *P. salmonis*, respectively (Fig. 1B). These results align with similar analyses in trout, where an increased expression of TLR5 was found 4 h post challenge with *Vibrio anguillarum*, a flagellated Gram-negative bacterium (Tsujita et al. 2004).

Moreover, TLR1 was chosen for analysis because *P. salmonis*, as a Gram-negative bacterium, possesses proteins and carbohydrates on its lipid membrane that can be recognized by both TLR1 and TLR22 (Palti 2011). The present results clearly evidenced a stimulation of TLR1 expression, which could be a consequence of stimulation with PRL following 2 h of incubation. Maximum expression was obtained at 4 h, reaching 12-fold more than the control group (Fig. 2A). Modulation of TLR1 was also found when incubation occurred conjointly with PRL and bacteria, although the result was similar to the PRL-only treatment (Fig. 2B).

Moreover, given the link between PRL and translocation of NF-κB from cytoplasm to the nucleus (Olavarría et al. 2010), it was of interest to determine the effect of PRL on IκBα. NF-κB is a transcription factor capable of activating the expression of certain genes, many of which are involved in innate immune response activation (Rothwarf & Karin 1999). In its inactive, non-stimulatory form, NF-κB is joined with its cytoplasmic repressor, IκBα (Israël 2000). For NF-κB to dissociate from its IκBα repressor in mammals, phosphorylation needs to occur, acting as a signal for ubiquitination and degradation in the proteasome, thus leaving the factor free for translocation to the nucleus (Maniatis 1999). Classically, IκBα is phosphorylated by IκK, an IκB kinase (Chen et al. 1996). However, under stimulation of other class I cytokines from the prolactin family (erythropoietin), IκB can be phosphorylated by other kinases such as JAK2 (Digicaylioglu & Lipton 2001). It is worth mentioning that in mammals, as well as in fish, the classic pathway described for PRL is the JAK/STAT pathway (Olavarría et al. 2010). The expression of IκBα over the course of the experiment (Fig. 3A) provides greater knowledge regarding the participation of NF-κB in signaling pathways for PRL, by clearly evidencing greater than 8-fold more transcripts when compared to the control group. When simultaneously administering PRL and *P. salmonis*, a greater effect was observed than that obtained for only PRL (Fig. 3B), indicating its potential application as an immunostimulant. Finally, one of the most notable effects occurred after stimulation with PRL at 2 h after infection, when expression of IκBα reached over 100-fold more than the control (Fig. 3C). Such results could provide initial evidence of PRL having immunostimulatory effects, even after infection has occurred. Along the same line, observed translocation of NF-κB in SHK-1 cells stimulated with PRL over the course of the experiment (Fig. 6) strongly reaffirms the obtained results, given that a gradual translocation of the factor occurred over time, and was inhibited when AG-490, an inhibitor of JAK2, was used. Given the clear relationship between the JAK/STAT pathway and PRL (Olavarría et al. 2010), the present study demonstrated that PRL stimulates the expression of TLR1 and TLR5M, IL-1β, and IκBα. The drastic decrease in the expression of IL-1β, at all concentrations of AG-490 used (Fig. 5), indicates that the course of action of the TLRs analyzed is probably JAK/STAT, although additional analyses are required to confirm this hypothesis.

The present results create new questions related to the possibility that JAK2 kinase, like IκK, is capable of phosphorylating IκBα and promoting the translocation of NF-κB to the nucleus, thus activating the transcription of genes related to the innate immune response. Finally, expression of IL-1β over time showed immunomodulatory effects of PRL on expression of the effector gene relevant in the immune response. Such results are in complete alignment with reports for other fish species, such as *S. aurata*, a species in which PRL also showed an immunostimulatory effect (Olavarría et al. 2010).

In conclusion, we found that PRL has immunostimulatory effects in SHK-1 cells of *S. salar* through the expression of TLR1 and TLR5M, as well as through IκBα and IL-1β. Likewise, our results demonstrated that PRL is capable of executing its immunostimulatory effects even after infection with *P. salmonis* has occurred. Taking these data into consideration, this study provides new evidence that PRL could have an alternative signaling pathway using JAK2 and NF-κB.

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