Prolactin regulates luminal bicarbonate secretion in the intestine of the sea bream (*Sparus auratus* L.).

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SHORT TITLE: PRL in intestinal bicarbonate secretion

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ABSTRACT

The pituitary hormone prolactin (PRL) is a pleiotropic endocrine factor that plays a major role in the regulation of ion balance in fish, with demonstrated actions mainly in the gills and kidney. The role of PRL in intestinal ion transport remains little studied. In marine fish, that have high drinking rates epithelial bicarbonate secretion in the intestine produces luminal carbonate aggregates believed to play a key role in water and ion homeostasis. The present study was designed to establish the putative role of PRL in the regulation of intestinal bicarbonate secretion (BCS) in a marine fish.

Basolateral addition of PRL to the anterior intestine of the sea bream mounted in Ussing chambers causes a rapid (<20 min) decrease of BCS measured by pH-stat. A clear inhibitory dose response curve is obtained, with a maximal inhibition of 60-65% of basal BCS. The threshold concentration of PRL with a significant effect on BCS is 10 $ng.ml^{-1}$, which is comparable with putative plasma levels in seawater fish. The effect of PRL on apical BCS is independent of the generation route for bicarbonate, as shown in a preparation devoid of basolateral HCO_3^- /CO_2 buffer. In addition, specific inhibitors of JAK2 (AG-490, 50µM), PI3K (LY-294002, 75µM) or MEK (U-012610, 10µM) cause a 50-70% reduction in the effect of PRL on BCS, and demonstrate the involvement of PRL receptors. In addition to rapid effects, PRL actions are mediated at a genomic level. Incubation of intestinal explants of anterior intestine of the sea bream in vitro for 3 hours demonstrates a specific effect of PRL on expression of SLC4A4 (Na^+-HCO_3^- co-transporter), but not on SLC26A6 or SLC26A3 (Cl^- / HCO_3^-- exchangers). We propose a new role for PRL in the regulation of BCS, an essential function for ion/water homeostasis in the intestine of marine fish.

KEY WORDS: Prolactin, intestine, epithelial transport, bicarbonate secretion, sea bream, JAK2, MEK, PI3K, SLC4A4, SLC26A6, SLC26A3

INTRODUCTION

The blood of marine teleosts has an osmolality of 300-370 mOsm/kg, while seawater has an osmolality of 1000 mOsm/kgH_2O. This difference in osmolality causes a continuous diffusive water loss by the gills in marine fish that accompanies the branchial process of ion secretion that removes excess plasma salts (Evans et al., 2005). Consequently, water replacement becomes of the highest importance to sustain body ionic regulation. Thus, marine teleosts are required to drink substantial amounts of seawater (Fuentes and Eddy, 1997), which once ingested undergoes an initial desalting step in the esophagus by selective absorption of NaCl (Hirano and Mayergostan, 1976; Parmelee and Renfro, 1983) to facilitate water absorption in the intestine.

An important physiological process related to intestinal fluid processing, and ultimately to water absorption, in the intestine of marine teleosts is the production of divalent carbonate aggregates within the intestinal lumen, which in addition to intestinal processing are a relevant part of the carbon cycling in the ocean (Wilson et al., 2009). The formation of carbonate aggregates, which takes place also in unfed fish, has at least two requirements: high calcium availability and high pH to drive the precipitation. The substrates calcium and/or magnesium are in high concentration in the intestinal lumen (i.e. ingested seawater) and in addition, epithelial bicarbonate secretion creates alkaline conditions (Faggio et al., 2011; Fuentes et al., 2010b; Grosell, 2011; Kurita et al., 2008; Walsh et al., 1991; Wilson and Grosell, 2003; Wilson et al., 2009; Wilson et al., 2002). Thus, secreted bicarbonate immobilizes divalent ions in the form of carbonate aggregates, reduces fluid osmolality, and ultimately favors osmotic water absorption. The mechanisms responsible for bicarbonate secretion are not completely understood, although the involvement of some transporters has been established. On the basolateral membrane the Na^+-HCO_3^- cotransporter (NBC) that belongs to the SLC4 gene family leads to accumulation of cellular HCO_3^- (Kurita et al., 2008; Taylor et al., 2010). In the apical membrane, Cl^-/HCO_3^- anion exchangers belonging to the sulfate permease family, SLC26, secrete HCO_3^- concomitantly with Cl^- absorption (Ando and Subramanyan, 1990; Grosell and Genz, 2006; Kurita et al., 2008). In the absence of a contribution from the transcellular route that is mediated by the action of basolateral SLC4, a good portion of the apical alkaline secretion still takes place (Fuentes et al., 2010b; Taylor et al., 2010) and is likely produced by hydration of the CO_2 in the enterocyte driven by carbonic anhydrase (Grosell et al., 2007). Bicarbonate transporters required for apical secretion are mostly non-electrogenic and rely on the electrogenic action of basolateral Na^+/K^+ ATPase (Grosell and Genz, 2006). The Na^+/K^+ ATPase extrudes $3Na^+$ in exchange for $2K^+$ and

establishes a strong cytosolic negative potential and low intracellular concentration of Na^+ (Skou, 1990; Skou and Esmann, 1992).

Bicarbonate secretion for luminal aggregate formation is specific to marine fish (Grosell, 2011) and does not occur in fresh water. In addition, Na^+/K^+ ATPase activity and gene expression are higher in the intestine of seawater fish than in freshwater fish (Cutler et al., 2000; Fuentes et al., 1997; Jensen et al., 1998; Seidelin and Madsen, 1999).

The pituitary hormone prolactin (PRL) is a pleiotropic endocrine factor involved in the regulation of water and electrolyte balance in most classes of vertebrates, including teleost fish (Bole-Feysot et al., 1998). Osmoregulatory actions of PRL in fish have been extensively reviewed (Manzon, 2002) and the importance of this hormone in fish osmoregulation was first demonstrated in 1950s by studies on the euryhaline teleost *Fundulus heteroclitus* (Manzon, 2002; Pickford and Phillips, 1959). These studies showed that fish were unable to survive in fresh water if subjected to hypophysectomy, while replacement therapy with PRL allowed survival by limiting haemodilution. The target for PRL action was later identified as the Na^+/K^+ ATPase (Pickfford et al., 1970). Since then, the regulatory action of PRL on gill Na^+/K^+ ATPase was established in a series of freshwater and euryhaline species such as *Salmo salar* (Tipsmark and Madsen, 2009), *Salmo trutta* (Seidelin and Madsen, 1999), *Oreochomis mossambicus* (Tipsmark et al., 2011), *Dicentrarchus labrax* (Varsamos et al., 2006) and *Sparus aurata* (Mancera et al., 2002).

We have recently demonstrated that intestinal bicarbonate secretion in marine fish is an endocrine regulated process (Fuentes et al., 2010b) with inhibitory actions of the parathyroid hormone-related protein (PTHrP) and stimulatory action of stanniocalcin (STC). PRL-receptors have been identified in the sea bream and the intestine is amongst the tissues with high expression of the receptor (Santos et al., 2001), even in seawater fish, and makes the tissue a likely target for PRL. Taking into consideration the likely importance of Na^+/K^+ ATPase on the electrogenic potential required for bicarbonate secretion, together with the established regulatory action of PRL in gill Na^+/K^+ ATPase, we hypothesized that PRL is involved in the regulation of BCS in marine fish intestine. The present study was designed to test this hypothesis using an *in vitro* model, the intestinal epithelium of the sea bream (*Sparus auratus*)

MATERIAL AND METHODS

Peptides and Chemicals

Ovine PRL (NIADDK-oPRL-21) was obtained from the National Institutes of Health

(Bethesda, MD, USA) and has previously been shown to be bioactive in the sea bream (Mancera et al., 2002). All chemicals were of the highest grade and were obtained from Sigma-Aldrich (Madrid, Spain) unless stated otherwise.

Animals

Sea bream juveniles (*Sparus auratus*) were obtained from commercial sources (CUPIMAR SA, Cadiz, Spain) and stocked at the Ramalhete Experimental Marine Station (CCMAR, University of Algarve). Fish were held in 1000-liter seawater tanks (density < 5 kg fish/tank), with flowing seawater (salinity 36 ppt; temperature 18 – 24 °C), under natural photoperiod and were fed once a day (10 AM) with 2 % body weight commercial dry pellets (Provimi, Portugal). All fish were fasted for 48 h before experimental manipulations. For tissue collection fish (200 - 300g body weight) were anesthetized with 2-phenoxyethanol (1 ml/l water, Sigma-Aldrich) and killed by decapitation.

All animal manipulations were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. All animal protocols were performed under license of Group-1 from the Direcção-Geral de Veterinária, Ministerio da Agricultura, do Dessevolvimento Rural e das Pescas (Portugal).

Intestinal bicarbonate secretion (BCS)

Fish were killed by decapitation and a section of anterior intestine (3-5 cm caudal from the pyloric caeca) was dissected out and washed in chilled saline. The intestinal portions were defatted, cleaned with fresh saline, and opened longitudinally to produce a flat sheet that was mounted on a tissue holder (P2413, 0.71 cm^2, Physiological Instruments, San Diego, US) and positioned between two half-chambers (P2400, Physiological Instruments, San Diego, US) containing 1.5 ml of physiological saline. Basolateral and apical saline were prepared as previously described (Fuentes et al., 2006; Fuentes et al., 2010b) to simulate in vivo like conditions and had the following composition, basolateral saline: 160 mM NaCl, 1 mM $MgSO_4$, 2 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 3 mM KCl, 5.5 mM Glucose, 5 mM Hepes (4-(2-Hydroxyethyl)piperazinyl-1-ethanesulfonic), pH = 7.800, gassed with 0.3 % CO_2 + 99.7 % O_2; apical saline: 88 mM NaCl, 9.5 mM $MgCl_2$, 3 mM KCl, 7.5 mM $CaCl_2$, 126.5 mM $MgSO_4$ and 1 mM Na_2HPO_4, gassed with 100% O_2. pH of apical saline was maintained constant to 7.800 by pH-Stat. Experiments in which $NaHCO_3$ was omitted from the basolateral saline for experimental purposes it was replaced by HEPES-Na (2-(4-(2-Hydroxyethyl)piperazinyl-1-ethanesulfonic acid sodium salt) in an equivalent concentration

(160 mM NaCl, 1 mM $MgSO_4$, 2 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 5 mM HEPES-Na, 3 mM KCl, 5.5 mM glucose and 5 mM HEPES; pH = 7.800) and continuously mixed and oxygenated by gently gassing with 100 % O_2. The temperature was maintained at 21-22 °C throughout the experiments.

All bioelectrical variables were monitored by means of Ag / AgCl electrodes (with tip asymmetry < 1 mV) connected to either side of the Ussing chamber with 3-mm-bore agar bridges (1 M KCl in 3% agar). Voltage (mvolts) was monitored by clamping of epithelia to 0 $\mu A/cm^2$ (VCC600 Physiologic Instruments, San Diego, US). Epithelial resistance (Rt, $\Omega.cm^2$) or tissue conductance (Gt, $mS.cm^{-2}$) were manually calculated by Ohm's law using the voltage deflection induced by a 10 μAmp pulse of 3 sec every minute. Bioelectrical parameters for each tissue were recorded after the tissue achieved a steady state, usually between 30 - 40 min after mounting in chambers. To allow pulsing for Rt calculations during the titration, the VCC 600 was grounded to the pH-Stat assembly.

Bicarbonate secretion was measured by means of pH-stat assembly consisting on an ABU80 microburette, a TT80 titration controller and a PHM84 meter (Radiometer Copenhagen) attached to a mini pH electrode (Hanna). Luminal saline was kept at a constant pH of 7.800 throughout the experiment by addition of acid titrant (2.5 mM HCl). Bicarbonate secretion ($nmol.h^{-1}.cm^{-2}$) was calculated from the volume of titrant added, the concentration of the titrant and surface area (cm^2).

Experiments were only performed with tissue if voltage and bicarbonate secretion were stable over an 1 h. Once a steady state was achieved bicarbonate secretion monitored for 30 min served as the control. Hormones or pharmacological compounds were added to Ussing chambers and tissue response monitored over an hour.

Short Circuit Current (Isc) measurement

The anterior intestine was collected, isolated and mounted as previously described (Fuentes et al., 2010b) on a tissue holder (P2413, 0.71 cm^2, Physiological Instruments, San Diego, US). The tissue was positioned between two half- chambers (P2400, Physiological Instruments, San Diego, US) containing 2 ml of the serosal physiological saline (160 mM NaCl, 1 mM $MgSO_4$, 2 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 3 mM KCl, 5.5 mM glucose and 5 mM HEPES, pH = 7.800). During the experiments the tissue was gassed with 0.3% CO_2 + 99.7% O_2 and the temperature maintained at 21-22 °C. All bioelectrical variables were monitored by means of Ag/AgCl electrodes (with tip asymmetry < 1 mV) connected to either

side of the Ussing chamber with 3mm bore agar bridges (KCl 1 M in 3 % agar). Short circuit current (Isc, μA/cm^2) was monitored by clamping of epithelia to 0 mV. Voltage clamping and current injections were performed by means of a DVC-1000 voltage clamp amplifier (WPI, Sarasota, US), or a VCCMC2 (Physiologic Instruments, San Diego, US). Bioelectrical parameters for each tissue were recorded after the tissue achieved a steady state usually between 30 - 40 min after mounting in the chambers, and prolactin (1000 ng. ml^{-1}) was added to the basolateral side and Isc monitored for 40 min thereafter.

Identification of PRL intracellular signaling pathways

The anterior intestine of sea bream was mounted on a tissue holder (P2413, 0.71 cm^2, Physiological Instruments, San Diego, US) and positioned between two half-chambers (P2400, Physiological Instruments, San Diego, US) with 1.5 ml of basolateral saline and 1.5 ml of apical saline. The temperature was maintained at 21-22 °C. After an initial control period of 30 min, inhibitor was added to the basolateral side of the Ussing chamber for 40 min. The Janus kinase-2 (JAK2) inhibitor AG490 (Tyrphostin, Sigma) was used at concentration 50 μM, mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor U0126 (Sigma) at concentration 10 μM and the phosphoinositide 3-kinase (PI3K) inhibitor LY-294002 at a concentration 75 μM. After pre-incubation with inhibitors, Prolactin (1000 ng/ml) was added to the basolateral side and the secretion of bicarbonate was monitored for an additional 40 min period.

Quantification of transporter gene expression

To establish effects of PRL on selected HCO_3^- transporter expression 3 h ex-vivo cultures were used. The expression of the basolateral Na^+- HCO_3^- co-transporter (SLC4A4) and the apical Cl^-/HCO_3^- exchangers SLC26A6 and the SLC26A3 were analyzed in response to different levels of PRL in the culture medium (0, 1, 10, 100 and 1000 ng. ml^{-1}). Fish were captured, decapitated, the abdominal cavity exposed and the intestinal tract collected into oxygenated saline: 160 mM NaCl, 1mM $MgSO_4$, 2 mM NaH_2PO_4, 1.5mM $CaCl_2$, 5mM $NaHCO_3$, 3mM KCl, 5.5mM Glucose, 5mM Hepes, and 10μl/ml antibiotics (penicillin 10.000 IU/ml + streptomycin 10.000 UG/ml; Sigma Aldrich, Spain), pH = 7.800; 0.3 % CO_2 + 99.7 % O_2). The anterior region of the sea bream intestine samples were flushed–out with saline, cut, open and flattened. Ten longitudinal sections of a similar size (20-30 mg) from the anterior intestine of the same individual were collected. Tissue explants (in duplicate) were

transferred to individual wells of a sterile 24-well plate, suspended in 2 ml saline in an incubation chamber with a controlled atmosphere (0.3 % CO_2 + 99.7 % O_2). Samples from the same fish were exposed to different concentrations of PRL (0, 1, 10, 100 and 1000 ng/ml) and incubated for 3 h. After incubation samples were placed in RNA later (Sigma Aldrich, Spain) and stored for 1 week at 4ºC until utilized.

Total RNA was extracted with the Total RNA Kit I (E.Z.N.A, Omega, US) following the manufacturers instructions and the quantity and quality assessed (Nanodrop 1000, Thermo Scientific, US). Prior to cDNA synthesis RNA was treated with DNase using a DNA-free Kit (Ambion, UK) following the supplier's instructions. Reverse transcription of RNA into cDNA was carried out using RevertAid First Strand cDNA Synthesises Kit (Fermentas) following the manufactures' instructions with 500 ng of Total RNA in a final reaction volume of 20 µl.

For primer design ion transporter sequences from sea bream were identified in the EST collection database in the National Center of Biotechnology (NCBI, http://blast.ncbi.nlm.nih.gov/) using BLASTn queries of known fish sequences. Multisequence alignments using ClustalX were used to confirm sequence identity. Specific primers were designed using Primer3 (http://frodo.wi.mit.edu/) running under the EBioX (http://www.ebioinformatics.org/) interface for Macintosh. Table 1 shows primer sequences, amplicon sizes and NCBI accession numbers of the ion transporter sequences analyzed.

Real-time qPCR amplifications were performed in duplicate with 75ng cDNA, 350 nM of forward and reverse primers and the reporter dye SsoFast EvaGreen Supermix (Bio Rad, US) using the iCycler iQ (BioRad, US). The amplification protocol was as follows: denaturing and enzyme activation step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 5 sec and 63 °C for 10 sec. At the end of amplification, a temperature-dependent dissociation step was carried out at 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s.

To estimate amplification effieciencies, a standard curve was generated for each primer pair from 10-fold serial dilutions (from 100 to $1x10^{-4}$ pg) of a pool of first-stranded cDNA template from all samples. Standard curves represented the cycle threshold value as a function of the logarithm of the number of copies generated, and was defined arbitrarily as one copy for the most diluted standard. All calibration curves exhibited correlation coefficients >0.99, and the corresponding real-time PCR efficiencies were >99 %. Normalization of expression for each target gene was performed using 18S ribosomal protein (18s) expression.

Statistics

Results are shown as means ± SE unless otherwise stated. After assessment of normality and

homogeneity of variances, difference between groups were established using Student's t-test, one-way analysis of variance (ANOVA) or ANOVA repeated measures followed by the post hoc Bonferroni test. All statistical analyses were performed using GraphPad Prism version 5.00 for Macintosh (GraphPad Software, San Diego, CA, USA). Groups were considered significantly different at $p < 0.05$, unless stated otherwise.

RESULTS

Effect of Prolactin on bicarbonate secretion and short circuit current

The basolateral addition of PRL (1000 ng.ml^{-1}) to a preparation of the anterior intestine of the sea bream resulted in a significant decrease ($p < 0.05$, one-way ANOVA, repeated measures) of BCS within 20 min and reached a minimum within 40 min (Figure 1A). The effects of PRL on BCS were achieved without modification of epithelial selectivity as shown by unchanged values of tissue resistance (Rt, Ω.cm^2; Figure 1B). Removal of PRL from the basolateral saline and washing of the preparation with PRL-free saline did not restore BCS to basal values, at least during 1 h of measurement (Figure 1C). Basolateral addition of PRL (1000 ng.ml^{-1}) did not evoke changes in short circuit current (Isc, µAmp/cm^2 , Figure 2).

Dose-effect of Prolactin in bicarbonate secretion

To establish if the effect of PRL on BCS in the anterior intestine of sea bream followed a typical dose response curve, increasing doses of PRL were added to Ussing preparations of the anterior intestine at 45 min intervals (Figure 3). PRL applied between 1 and 1000 ng.ml^{-1} evoked a dose-dependent reduction of BCS in the anterior intestine of the sea bream (Figure 3). The lowest dose at which a statistically significant effect of PRL on BCS was detected was 10 ng.ml^{-1} ($p < 0.05$, one-way ANOVA, repeated measures) and higher doses induced further decrease in intestinal BCS. For clarity the data are presented as dose-response inhibition of BCS by PRL, and maximal inhibitory effects reached 60-65% of basal BCS and were obtained with doses of PRL between 100-1000 ng. ml^{-1} (Figure 3B).

Inhibition of basolateral Na^+/K^+ ATPase overrides Prolactin effects

Figure 4 compares the inhibitory effect of PRL (1000 ng. ml^{-1}), Ouabain (1 mM) or a combination of both on BCS in the anterior intestine of the sea bream. With the addition of basolateral PRL alone to anterior intestine preparations of the sea bream a plateau was reached from circa 40 min onwards and showed stable inhibition of 50 % BCS. Addition of 1 mM of the Na^+/K^+ ATPase inhibitor Ouabain, alone or in combination with basolateral PRL

induced a continuous inhibition of intestinal BCS basal secretion that reached circa 70 % at the end of the experimental period (60 min) and was significantly higher than PRL alone ($p < 0.05$, one-way ANOVA; Figure 4).

Prolactin inhibitory effect on BCS is cellular and transcellular

The response of BCS to basolateral PRL was tested in the presence or absence of basolateral HCO_3^- /CO_2 (Figure 5A and 5B, respectively). In both cases the application of PRL (1000 ng./ml^{-1}) had an inhibitory effect on BCS ($p < 0.05$, Student's t test) and the magnitude of inhibition was similar in both experiments (66-68% in relation to basal secretion). However, the absolute effect of PRL in the presence of basolateral HCO_3^- /CO_2 was significantly higher than PRL alone ($p < 0.05$, Student's t-test; Figure 5C). The inhibitory effect of PRL on BCS was proportional to the transcellular/endogenous generation of bicarbonate for apical secretion

Prolactin effect on BCS are mediated by PRL -receptors

Several inhibitors of intracellular signaling cascades were used to determine the specific intracellular signaling pathways modified when PRL activates its receptor in the sea bream intestine to bring about its effect on BCS. In the presence of a JAK2 pathway inhibitor (AG-490, 50 µM) the inhibitory effects of PRL (1000 ng.ml^{-1}) on BCS were significantly reduced by 60 % ($p<0.05$, Student's t-test). Interestingly, 75 µM LY-294002, a specific PI3K inhibitor and 10 µM U-0126, a specific MEK inhibitor, produced a similar significant reduction ($p < 0.05$, Student's t-test) in the effect of PRL on BCS in the anterior intestine of the sea bream (Figure 6). The results indicate that the Jak2, PI3K and MEK intracellular signaling mediate the effect of PRL on BCS in the intestine of the sea bream.

HCO_3^- transporter gene expression in response to prolactin.

PRL at 10-1000 ng.ml^{-1} caused a statistically significant reduction in *SLC4A4* expression compared to untreated anterior intestine (Figure 7A; $p < 0.05$, One-way ANOVA, repeated measures). No significant effects were observed in the expression of apical *SLC26A6* and *SLC26A3* in response to PRL (Figure 7B,C).

DISCUSSION

The present study establishes a role for PRL in the regulation of BCS in the intestine of marine fish. The lines of evidence provided are as follows: BCS in the intestine of the sea

bream is sensitive to physiological levels (ng) of PRL, the effect of PRL follows a typical dose-response curve and the effect of PRL on BCS is reduced by blockade of PRL intracellular signaling of JAK2, MEK or PI3K.

In the marine sea bream PRL-receptor transcripts and protein are highly abundant in the intestine (Santos et al., 2001), indicating that it is a likely target for PRL although its function at this site has never been described. In the present study, we report the inhibitory action of PRL on intestinal BCS in the intestine of the sea bream. The action is rapid, as measured by pH-stat in Ussing chambers and reaches a plateau within 40 minutes. It also takes place without alteration of tissue barrier function, expressed electrically by Rt. Removal of PRL from the basolateral chamber did not restore BCS to values obtained previous to PRL treatment (1000 ng. ml^{-1}) within 3.5 h, the time period for maintenance of vital conditions in *in vitro* sea bream intestine preparations (Fuentes et al., 2006), and values remained stable until the end of experiments. The rapid time frame of the response to PRL is indicative that non-genomic pathways are responsible for this inhibitory action.

The concentration of PRL in fish plasma both in freshwater and seawater ranges is in the low ng. ml^{-1} range. In *Oreochromis mossambicus* and *O. niloticus* the range of plasma PRL is 5-20 $ng.ml^{-1}$ during short-term (under 24 hours) transfers between salinities (Breves et al., 2010) and in seawater-adapted *O. mossambicus*is is 3.4 $ng.ml^{-1}$ (Takahashi et al., 2007). In freshwater cannulated brown trout (*Salmo trutta*) plasma PRL levels are in the 0-30 $ng.ml^{-1}$ range (Waring et al., 1996) and 10-15 $ng.ml^{-1}$ in freshwater rainbow trout (*Salmo gairdneri*) and 3-5 $ng.ml^{-1}$ in seawater adapted trout (Prunet et al., 1985). Circulating plasma PRL levels in the sea bream have not been reported. However, based on the secretion pattern and pituitary content (Brinca et al., 2003; Fuentes et al., 2010a) the levels of PRL in sea bream plasma are likely to be within the range described for other species. The BCS responses of the anterior intestine to basolateral PRL revealed a significant threshold effect at the physiological level of 10 ng. ml^{-1}, with inhibition of BCS in the range of 40% (Figure 2). An inhibitory plateau in excess of 60 %was reached at levels $\geq$ 100 ng. ml^{-1} PRL. This result reveals the likely importance of PRL in the regulation of BCS *in vivo*. Even the lower plasma concentrations of PRL are expected to have a direct regulatory effect on BCS and indirectly on availability of water for intestinal absorption, with an end-result of increased plasma osmolality.

Luminal secretion of bicarbonate in the enterocyte of marine fish is the result of transcellular transport and endogenous generation of HCO_3^- from CO_2. The presence of a basolateral Na^+/HCO_3^- cotransporter (SLC4A4) and apical Cl^-/HCO_3^- exchangers (Kurita et al., 2008; Taylor et al., 2010) suggests that bicarbonate could be routed at a transcellular levels. However, in the absence of basolateral bicarbonate, a good portion of alkaline secretion still takes place (Fuentes et al., 2010b; Taylor et al., 2010), that is likely produced by hydration of the CO_2 in the intestinal epithelial cell by the action of carbonic anhydrase (Grosell et al., 2007). The values of intestinal BCS obtained in this study in the sea bream by pH-stat methods are in keeping with the range previously described in the same species using the double titration method (Fuentes et al., 2010b). In the conditions used for most of the experiments in this study (0.3% CO_2-5 mM HCO_3^- in the basolateral saline) only about 30% of BCS is likely driven via transcellular pathways, while the remainder is likely (Grosell et al., 2007) produced by hydration of CO_2 in the enterocyte (shown in CO_2/HCO_3^- free basolateral saline). In these conditions, it seems likely that the inhibitory effects of PRL on luminal BCS in the sea bream intestine are mediated by both transcellular and cellular mechanisms. This suggestion is supported by the significant effects of PRL on BCS in the Ussing chamber, both in the presence and the absence of basolateral HCO_3^-/CO_2 (Figure 4).

The mechanisms involved in BCS regulated by PRL in the intestine of the sea bream have not been definitely established. The link between PRL and Na^+/K^+ ATPase (Mancera et al., 2002; Tipsmark et al., 2011) was tested using the specific Na^+/K^+ ATPase inhibitor, ouabain, and resulted in a 70% inhibition of total BCS. In the anterior intestine of the Gulf toadfish Ouabain has a similar effect (Grosell and Genz, 2006). The effect of ouabain was explained by the reduction of the basolateral Na^+ gradient, that is essential to drive H^+ extrusion via a sodium proton exchanger (NHE) required for apical BCS (Grosell and Genz, 2006). The inhibitory effect of ouabain would account for a reduction in apical BCS derived from transcellular and cellular HCO_3^-, although this possibility was not tested in the present study. In the anterior intestine of the sea bream, the inhibition of apical BCS in response to ouabain is significantly higher than the effect of PRL alone. Furthermore, PRL added in combination with ouabain failed to induce additional inhibition of apical BCS. Taken together these results indicate that ouabain likely offsets the effect of PRL on the Na^+/K^+ ATPase. However, the reduction caused by ouabain on the basolateral Na^+ gradient required for apical BCS would mask effects of PRL on additional mechanisms. Interestingly, PRL and ouabain have different effects in voltage clamped preparations of anterior intestine in Ussing chambers. While no

effect is observed in response to PRL, ouabain totally abolished the absorptive current. This result may indicate that the effects of PRL are mediated via a non-electrogenic transporter. A recent study in the Gulf toadfish (Taylor et al., 2010) proposed the basolateral Na^+- HCO_3^- co-transporter (SLC4A4) to be a limiting step for apical BCS in the marine fish intestine. It is tempting to speculate that PRL has a regulatory action on the basolateral SLC4A4, as indicated by the genomic effect of PRL on the expression of *SLC4A4* gene but not on the expression of the apical anion transporters *SLC26A6* and *SLC26A3* genes (Figure 6). However, the inhibitory effect of PRL on BCS in preparations devoid of basolateral HCO_3^-/CO_2 points to PRL effects on other important mechanisms.

In higher vertebrates PRL-receptors signal via specific intracellular cascades involving the JAK2 pathway, PI3K, and/or MEK, depending on the tissues analyzed (Al-Sakkaf et al., 1997; Jantarajit et al., 2007; Piccoletti et al., 1994; Yamauchi et al., 1998). In fish, despite the evidence showing physiological actions of PRL in osmoregulation, little effort has been devoted to study PRL intracellular cascades. In our *in vitro* model of anterior intestine, PRL effects on BCS were significantly reduced by the action of the JAK2, PI3K and MEK inhibitors. This indicates that the effects of PRL on BCS are specifically mediated by PRL-receptors. In Caco-2 cell line monolayers and in rat duodenum, PRL stimulated transepithelial calcium movements are mediated by the actions of PI3K, but not by MEK or JAK2 (Jantarajit et al., 2007), while in the mammary gland cell line HC11, JAK2 mediates the actions of PRL in Cl^- transport (Selvaraj et al., 2000). Although further studies are required, it appears that the action of PRL on BCS in the anterior intestine of the sea bream is the result of signaling through the 3 intracellular pathways. Whether this biological action of PRL takes place via the previously characterized sea bream PRL receptor (Santos et al., 2001), or if multiple receptors are involved requires further investigation. More than a single functional receptor has been characterized in other fish, such as tilapia (Fiol et al., 2009)

In a previous study (Fuentes et al., 2010b) it was demonstrated that the calcitropic hormones PTHrP and STC had a significant role in the regulation of BCS and that it opposed the effects of both factors on net calcium transport in the intestine of the sea bream, thus modulating intestinal aggregate formation and water availability for absorption. The present study established that PRL has an osmoregulatory function in the intestine of marine fish compatible with its known freshwater adapting actions. Sustained intestinal BCS is a special feature of the intestine of marine fish directed at enabling water absorption and PRL actions

down-regulate this process. The effects are rapid, compatible with plasma circulating levels of PRL in freshwater and seawater fish and in light of the involvement of JAK2, MEK or PI3K intracellular pathways likely mediated by PRL receptors. In addition to its rapid effects, PRL regulates the expression of intestinal *SLC4A4*, proposed to be the rate limiting-step (Taylor et al., 2010) for apical BCS in the marine fish intestine. Based on the evidences from the present study a new functional role for PRL is proposed, namely, regulation of BCS in the intestine of marine fish, which is an essential function in ion/water homeostasis. It will be important to establish if other endocrine factors directly regulate intestinal BCS and whether calcium sensing (e.g. calcitropic factors) or osmosensing (e.g. PRL) are the predominant signal for regulation of this process

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Table 1. Primers for qPCR

Gene	Sequence (5'to 3`)	Amplicon (bp)	Accession No
SLC26A3	FW-ATCTCGGCTCTGAAGGGACT RV-GAGCATTTCTGTCCCTGCTC	162	AM973894
SLC26A6	FW-GCGGGACTGTTCAGCGGAGG RV-TGCGAACACGCCTGAACGGCA	176	FM155691.1
SLC4A4	FW-ACCTTCATGCCACCGCAGGG RV-CGCCGCCGCCGATAACTCTT	128	FM157528.1
18S	FW-AACCAGACAAATCGCTCCAC RV-CCTGCGGCTTAATTTGACTC	139	AY993930

FIGURE LEGENDS

Figure 1. A) Bicarbonate secretion (BCS, nmol.h^{-1}.cm^{-2}) as measured by pH-Stat and B) tissue resistance (Rt, Ω.cm2) in the anterior intestine of the sea bream in response to ovine prolactin (PRL, 1000 ng. ml^{-1}) applied to the basolateral side of the Ussing chambers. C) Effect of removal of prolactin from the basolateral saline and replacement with PRL-free basolateral saline (wash) on bicarbonate secretion. Each point represents mean ± SEM (n = 7). Asterisks represent significant differences from control periods ($p < 0.05$, one-way ANOVA, repeated measures)

Figure 2. Short-circuit current (Isc, µA.cm^{-2}) in the anterior intestine of the sea bream in response to ovine prolactin (PRL, 1000 ng. ml^{-1}) or ouabain (OUA, 1 mM) applied to the basolateral side of the Ussing chambers. Each bar represents mean ± SEM (n = 5). Asterisks represent significant differences from basal values ($p < 0.05$, one-way ANOVA, repeated measures)

Figure 3. A) Bicarbonate secretion (BCS, nmol.h^{-1}.cm^{-2}) as measured by pH-Stat in the anterior intestine of the sea bream in response to increasing concentrations of ovine prolactin (0, 1, 10, 100 and 1000 ng.ml^{-1}) applied to the basolateral side of the Ussing chambers. B) shows the inhibitory effect of each dose relative to basal values (% of inhibition). Each column represents mean ± SEM (n = 4). Asterisks represent significant differences from basal values ($p < 0.05$, one-way ANOVA, repeated measures)

Figure 4. Bicarbonate secretion (BCS, nmol.h^{-1}.cm^{-2}) as measured by pH-Stat in the anterior intestine of the sea bream. Results are shown as percentage of control values after 60 min basolateral exposure to ovine prolactin alone (PRL, 1000 ng.ml^{-1}), ouabain alone (OUA, 1 mM) or a combination of ovine prolactin and ouabain (PRL+ OUA, 1000 ng.ml^{-1} + 1 mM, respectively). Each bar represents mean ± SEM (n = 5-7). Different letters denote significantly different groups ($p < 0.05$, one-way ANOVA).

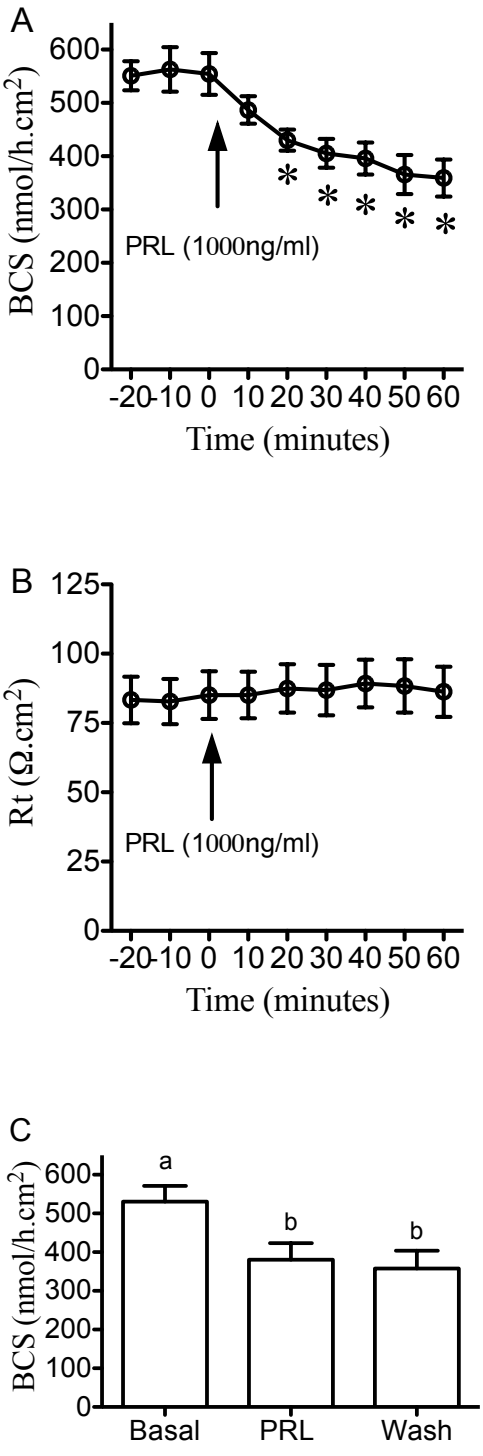
Figure 5. Bicarbonate secretion (BCS, nmol.h^{-1}.cm^{-2}) as measured by pH-Stat in the anterior intestine of the sea bream in response to ovine prolactin (PRL, 1000 ng.ml^{-1}) A) in the presence or B) absence basolateral HCO_3^-/CO_2. C) PRL-dependent bicarbonate secretion in

experiments A) and B). Each bar represents mean ± SEM (n = 7). Asterisks represent significant differences between treatments ($p < 0.05$, Student's t-test).

Figure 6. PRL-dependent bicarbonate secretion (BCS, $nmol.h^{-1}.cm^{-2}$) as measured by pH-Stat in the anterior intestine of the sea bream in response to ovine prolactin (PRL, 1000 $ng.ml^{-1}$) in the absence (left bar) or presence (right bar) of a Jak2 inhibitor (AG490, 50μM), a MEK inhibitor (U0126, 10μM) or a PI3K inhibitor (LY294002, 75μM). Each bar represents mean ± SEM (n = 5) Asterisks represent significant decrease of Prolactin effect in the presence of inhibitors ($p < 0.05$, Student's t-test).

Figure 7. A) *SLC4A4*, B) *SLC26A6* and C) *SLC26A3* gene expression relative to *18S* as measured by qPCR in anterior intestine of the sea bream after *in vitro* culture for 3 h in the absence (0) or presence of prolactin (PRL: 1, 10, 100 and 1000 $ng.ml^{-1}$). Each bar represents mean ± SEM (n = 6). Asterisks indicate significant differences from control ($p < 0.05$, one-way ANOVA, repeated measures)

Figure 1. Ferlazzo, Carvalho et al



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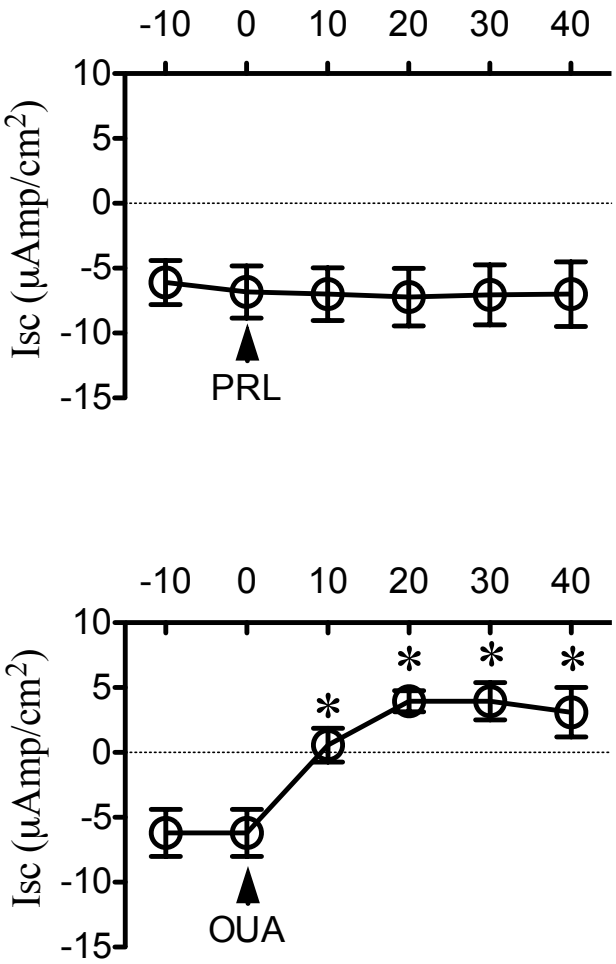

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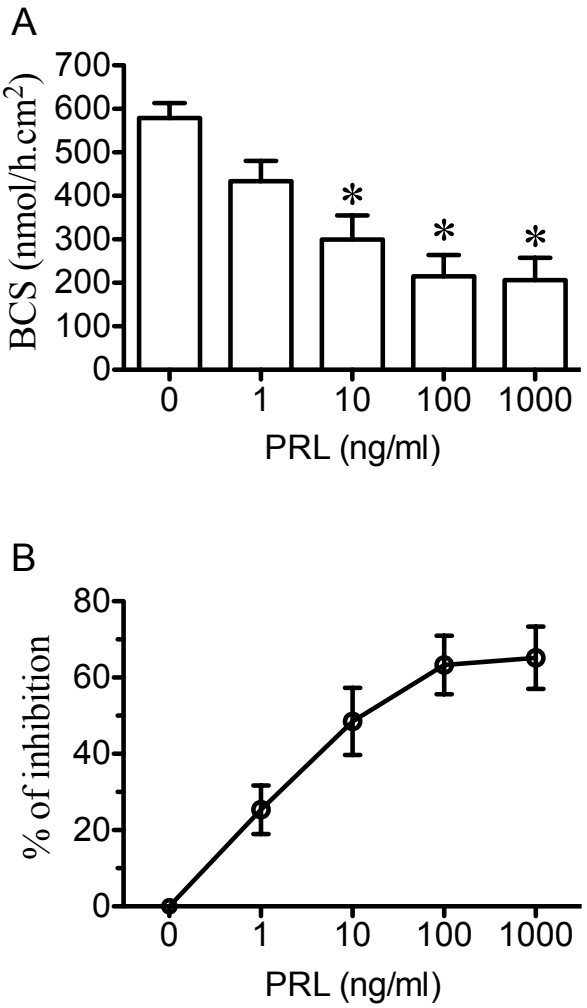

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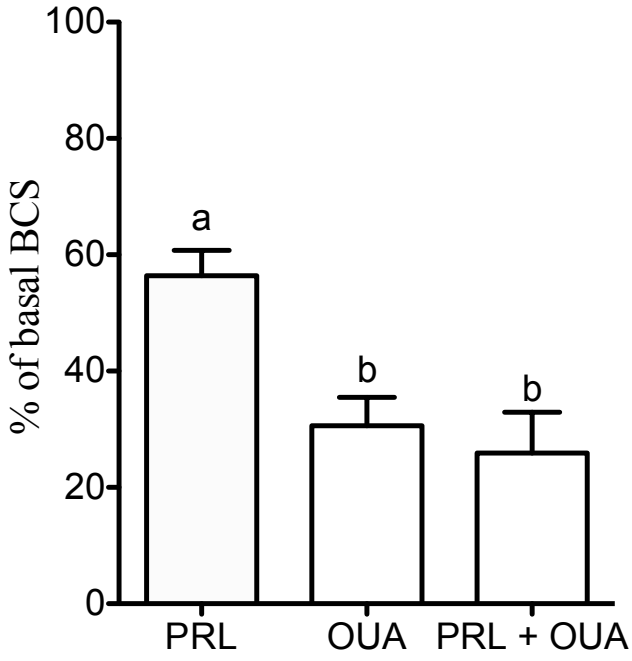

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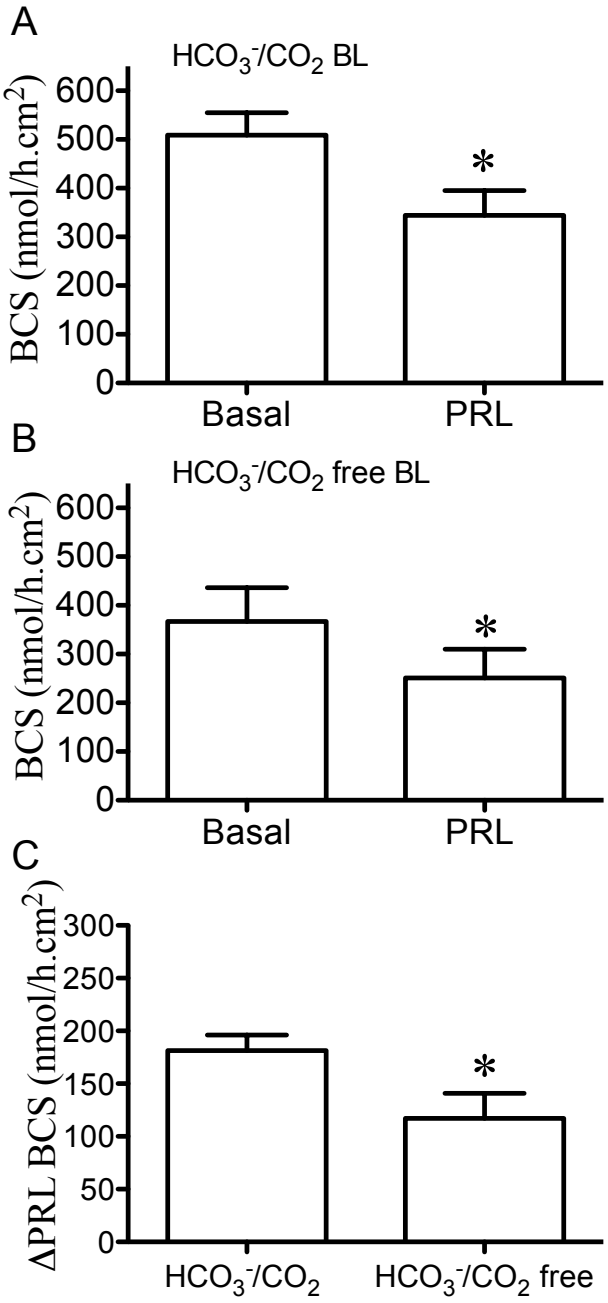

Figure 6. Ferlazzo, Carvalho et al

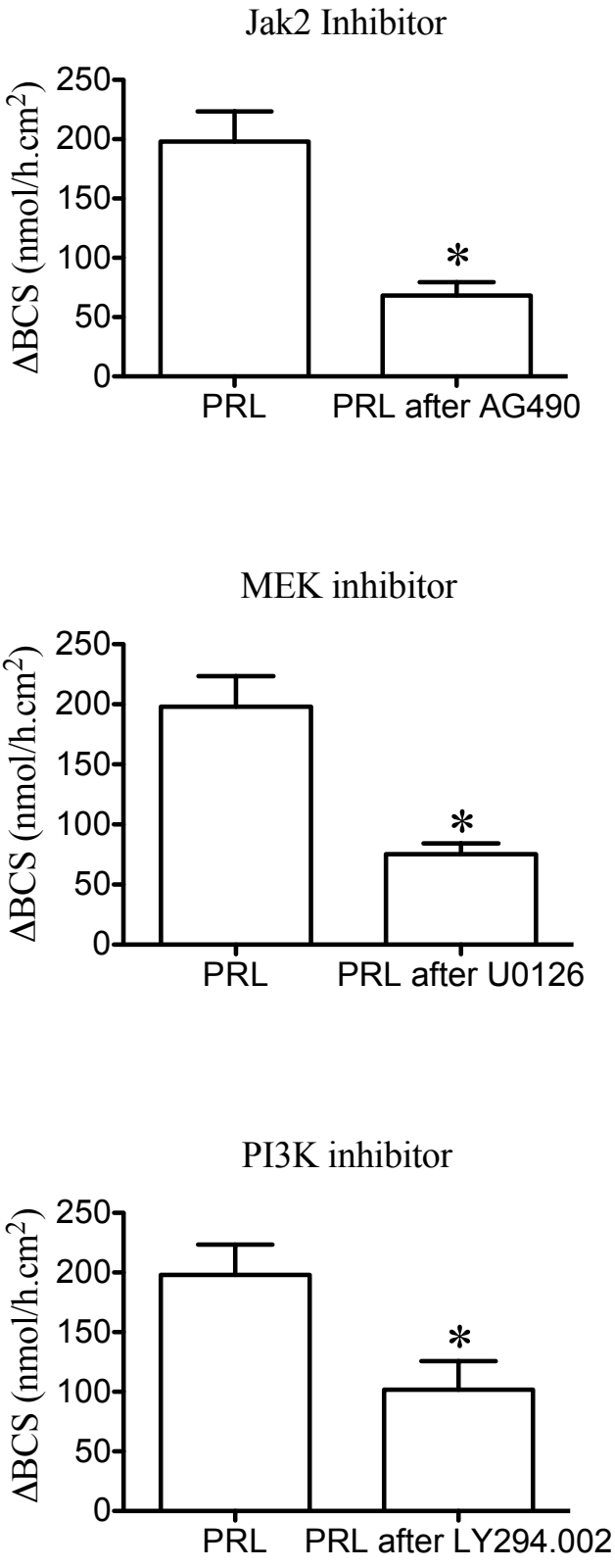

Figure 7. Ferlazzo, Carvalho et al