Prolactin inhibits osteoclastic activity in the goldfish scale: A novel direct action of prolactin in teleosts

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Prolactin Inhibits Osteoclastic Activity in the Goldfish Scale: A Novel Direct Action of Prolactin in Teleosts

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In teleosts, prolactin is involved in calcium regulation, but its role in scale/bone metabolism is unclear. Using the in-vitro system with goldfish scales developed recently, we explored the effects of teleost prolactin, growth hormone, and somatolactin on osteoclasts and osteoblasts. Addition of prolactin at concentrations of 0.01–100 ng/ml reduced osteoclastic activity, partly via osteoclast apoptosis, after 6–18 h incubation. Conversely, growth hormone and somatolactin at a concentration of 100 ng/ml increased osteoclastic activity after 18 h incubation, indicating the specificity of the inhibitory effect of prolactin on osteoclastic activity. On the other hand, these three hormones promoted osteoblastic activity at concentrations of 10–100 ng/ml. The results from this study are the first demonstration of direct effects of prolactin on scale/bone metabolism and osteoclastic activity in a teleost.

Key words: prolactin, growth hormone, somatolactin, osteoblast, osteoclast, calcium metabolism, scale

INTRODUCTION

The importance of the anterior pituitary hormone prolactin (PRL) in vertebrates is evident from its role in a wide spectrum of functions that include reproduction (or parental behavior), osmoregulation, and immunomodulation (see Bole-Feysot et al., 1998; Sakamoto et al., 2003; Harris et al., 2004). In teleost fishes, PRL also has hypercalcemic effects, mainly by influencing the uptake of calcium from the external environment (Flik et al., 1994; Chakraborti and Mukherjee, 1995; Seale et al., 2003). On the other hand, the role of PRL in calcium turnover in the teleost calcified tissues, bone and scale, remains largely unclear, although PRL influences bone metabolism in mammals by acting directly on osteoblasts (Clément-Lacroix et al., 1999; Coss et al., 2000; Seriwatanachai et al., 2008) and chondrocytes (Zermeño et al., 2006), and through the activation of synovial cell functions (Nagafuchi et al., 1999). In tilapia, bone density was shown to be increased by in-vivo treament with ovine PRL (Flik et al., 1986), which binds equally to both growth hormone (GH) receptors and PRL receptors in this species (Prunet and Auperin, 1994).

Teleost scales also contain osteoclasts and osteoblasts (Yamada, 1971; Bereiter-Hahn and Zylberberg, 1993; Yoshikubo et al., 2005; Suzuki et al., 2007), and the scales, rather than the body skeleton, jaws, or otoliths, appear to be an internal calcium reservoir, judging from a $^{45}Ca^{2+}$-labelling study of calcified tissues in goldfish and killifish (Mugiya and Watabe, 1977). In goldfish scales, the osteoclasts are of the multinucleated, active type that shows tartrate-resistant acid phosphatase (TRAP) staining (Suzuki et al., 2000) and in-situ hybridization with a cathepsin K probe (Azuma et al., 2007). In addition, components of the bone matrix, including type-I collagen (Zylberberg et al., 1992), bone γ-carboxyglutamic acid protein (Nishimoto et al., 1992), osteonectin (Lehane et al., 1999), and hydroxyapatite (Onozato and Watabe, 1979), are present in the scales.

In this context, we explored the direct effects of PRL on osteoclasts and osteoblasts in the scales of mature female goldfish by using a recently developed in-vitro system (Suzuki et al., 2000; Suzuki and Hattori, 2002). In addition,

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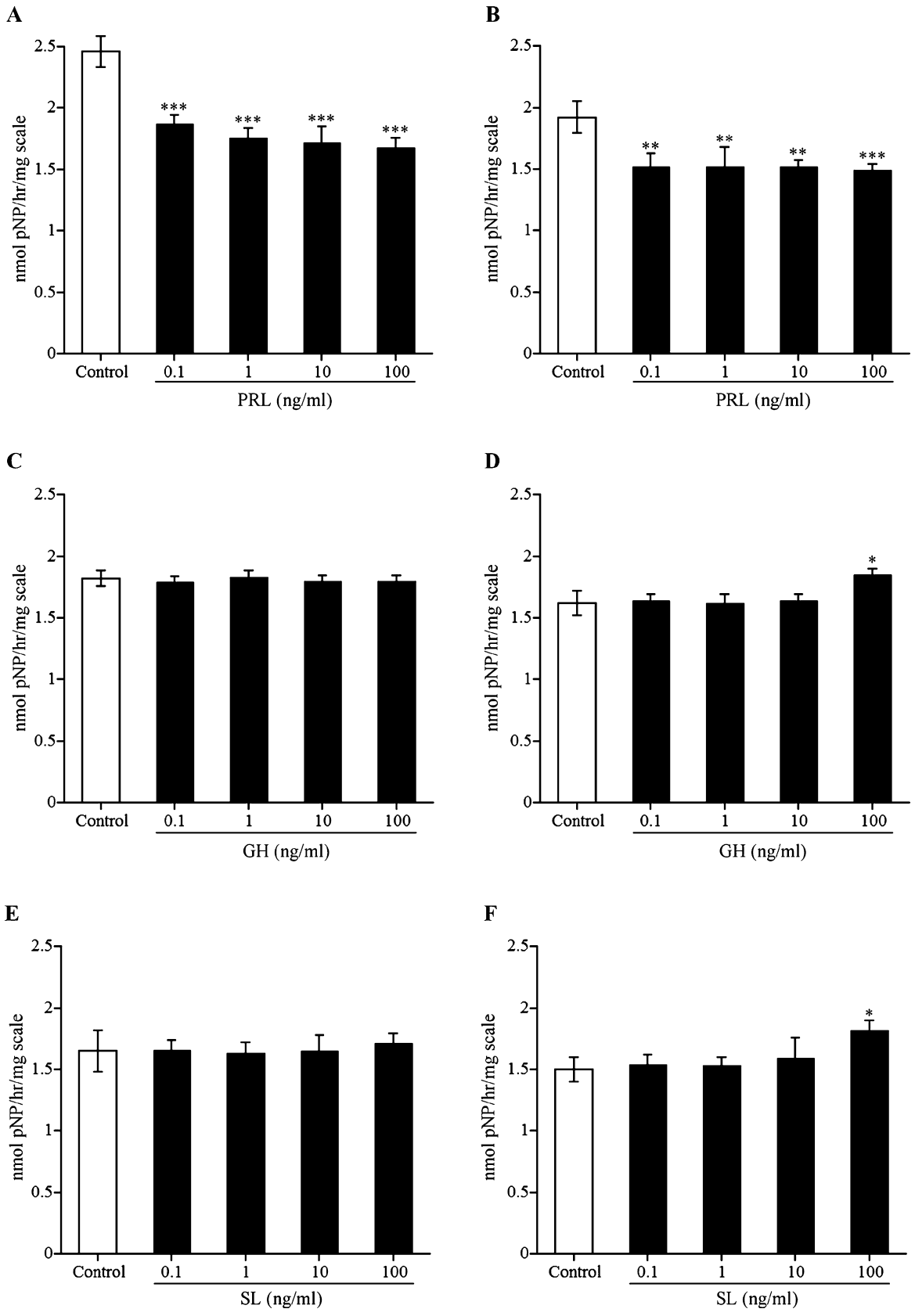

Fig. 1. Effects of PRL, GH, and SL (0.1 to 100 ng/ml) on TRAP activity in cultured goldfish scales after 6 h **(A, C, E)** and 18 h **(B, D, F)** of incubation. Values are mean±SEM (N=8). *, **, and *** indicate significant differences at $P<0.05$, 0.01, and 0.001, respectively, from the values in the control scales (ANOVA with Dunnett's post-hoc test).

we compared the actions of PRL with those of GH and somatolactin (SL), other members of a hormone family sharing a common ancestral gene with PRL in teleosts (Rand-Weaver et al., 1993). Here we report that PRL acts specifically on goldfish scales to reduce osteoclastic activity and promote osteoblastic activity. These findings indicate for the first time that PRL plays a direct role in the regulation of scale/bone metabolism in teleosts.

MATERIALS AND METHODS

Animals

A previous study using goldfish (Suzuki et al., 2000; Suzuki and Hattori, 2002) indicated that the sensitivity for calcemic hormones was highest in mature females. Therefore, mature female goldfish (*Carassius auratus*, 30–50 g in weight) were purchased from Higashikawa Fish Farm (Yamatokoriyama, Japan) as previously described (Suzuki et al., 2000). As needed, goldfish were anesthetized in tricaine methanesulfonate. All procedures were approved by the Okayama University Committee in accordance with national guidelines.

Primary scale culture

As previously described (Suzuki and Hattori, 2002), scales were removed from goldfish and incubated at 15°C in Eagle's modified minimum essential medium (MEM; ICN Biomedicals Inc., OH, USA) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals, Inc., OH, USA) with or without the addition of chum salmon PRL, GH, or SL. These salmonid hormones (Kawauchi et al., 1986; Yasuda et al., 1986; Rand-Weaver, 1993) exhibit approximately 70% amino-acid identities to the goldfish counterparts (Chan et al., 1996; Law et al., 1996; Cheng et al., 1997) and high specificities to their respective receptors in fishes (Prunet and Auperin, 1994; Tse et al., 2000; Lee et al., 2001; Fukada et al., 2005). For each comparison between treatment and control groups, scales were collected from a single fish. Scales were fixed for 2 h in cold 10% formalin in 0.05 M cacodylate buffer (pH 7.4), rinsed and kept in 0.05 M cacodylate buffer at 4°C until the analyses.

Assays of osteoclastic and osteoblastic activities

In our system, the activities of both osteoclasts and osteoblasts were detected with TRAP and alkaline phosphatase (ALP) as respective markers (Suzuki and Hattori, 2002), as similarly utilized for determination of the effects of particular hormones on osteoclasts and osteoblasts in mammals (Veas, 1988; Noda et al., 2005). We detected the respective enzyme activity from individual scales by transferring each scale into a well of a 96-well microplate for incubation.

For TRAP activity, scales were incubated at 20°C for 60 min in 200 μl of 100 mM sodium acetate buffer, pH 5.3, containing 20 mM tartrate and 10 mM para-nitrophenyl-phosphate. For ALP activity, the buffer was 100 mM Tris-HCl, pH 9.5, containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$. Color development was quantified by absorption at 405 nm.

Analyses of apoptotic osteoclasts

To examine the possible involvement of apoptosis in the inhibition of osteoclastic activity by PRL (see Figs. 1 and 2), osteoclasts were induced by the autotransplantation of scales in goldfish (our unpublished results). The collected scales were intramuscularly autotransplanted, and the fish were kept in fresh water containing antibiotic (Green F Gold, Sanei Co. Ltd., Tokyo, Japan) for 7 days. Thereafter, the transplanted scales were removed, cut into halves, and cultured as described above with or without salmon PRL (10 ng/ml). After 6 h incubation, the scale halves were fixed as above, and TRAP staining was performed by the methods of Cole and Walters (1987). After TRAP staining, DNA fragmentation associated with apoptosis was detected by the TUNEL method of Gavrieli et al. (1992) using an In Situ Cell Death Detection Kit (Roche, Tokyo, Japan; Takahashi et al., 2006a, b, 2007). The TRAP-stained samples were washed twice in 100 mM Tris-HCl buffer, pH 7.6, containing 150 mM sodium chloride and 0.1% Tween 20 (TBST), and fixed in methanol at –20°C for≥24 h. To rehydrate the samples following methanol fixation, each scale was washed in TBST four times at room temperature for 15 min. After microwave irradiation, the samples were transferred to TUNEL buffer (25 mM Tris-HCl, pH 7.6, containing 200 mM sodium cacodylate, 5 mM cobalt chloride and 0.25% bovine serum albumin), and incubated overnight at 4°C. After washing in TUNEL buffer for 30 min at room temperature, the scales were incubated at 37°C for 4 h with TdT and fluorescein-labeled dUTP. The reaction was terminated by transferring the scales to TBST for 15 min and mounted on a glass slide with a coverslip. The specimens were examined with a fluorescence microscope (EFDA2 with a 100-W Hg light source; Nikon, Tokyo, Japan) equipped with a chilled CCD camera (600CL, Pixera Co., Los Gatos, CA, USA). The excitation, dichroic, and emission filters were the EX 420–490, DM 510, and BA 520, respectively. The omission of TdT gave completely negative results. The ratio of TUNEL-positive osteoclasts per total osteoclasts was quantified at 200X magnification by image analysis (Studio 3.0, Pixera Co., Los Gatos, CA, USA). Five to seven osteoclasts were examined for each scale piece (N=11).

RESULTS

Effects of PRL, GH, and SL on osteoclastic activity in scales

Fig. 1 shows the effect of PRL, GH, and SL on scale TRAP activity as an osteoclastic marker in primary culture. PRL at concentrations of 0.1–100 ng/ml reduced TRAP activity by ~20% after 6 h and 18 h incubation. Even at 10 pg/ml, PRL was effective in reducing osteoclastic activity after 6 h (Fig. 2). In contrast, GH and SL increased TRAP activity by ~10% only at a concentration of 100 ng/ml after 18 h incubation; there was no significant effect after 6 h different from that by PRL. Increased concentrations of each

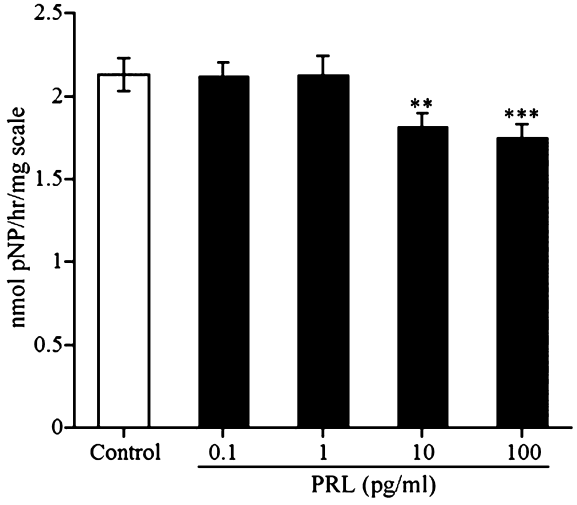

Fig. 2. Effects of PRL (0.1 to 100 pg/ml) on TRAP activity in cultured goldfish scales after 6 h of incubation. Values are mean±SEM (N=8). ** and *** indicate statistically significant differences at $P<0.01$ and 0.001, respectively, from the values in the control scales (ANOVA with Dunnett's post-hoc test).

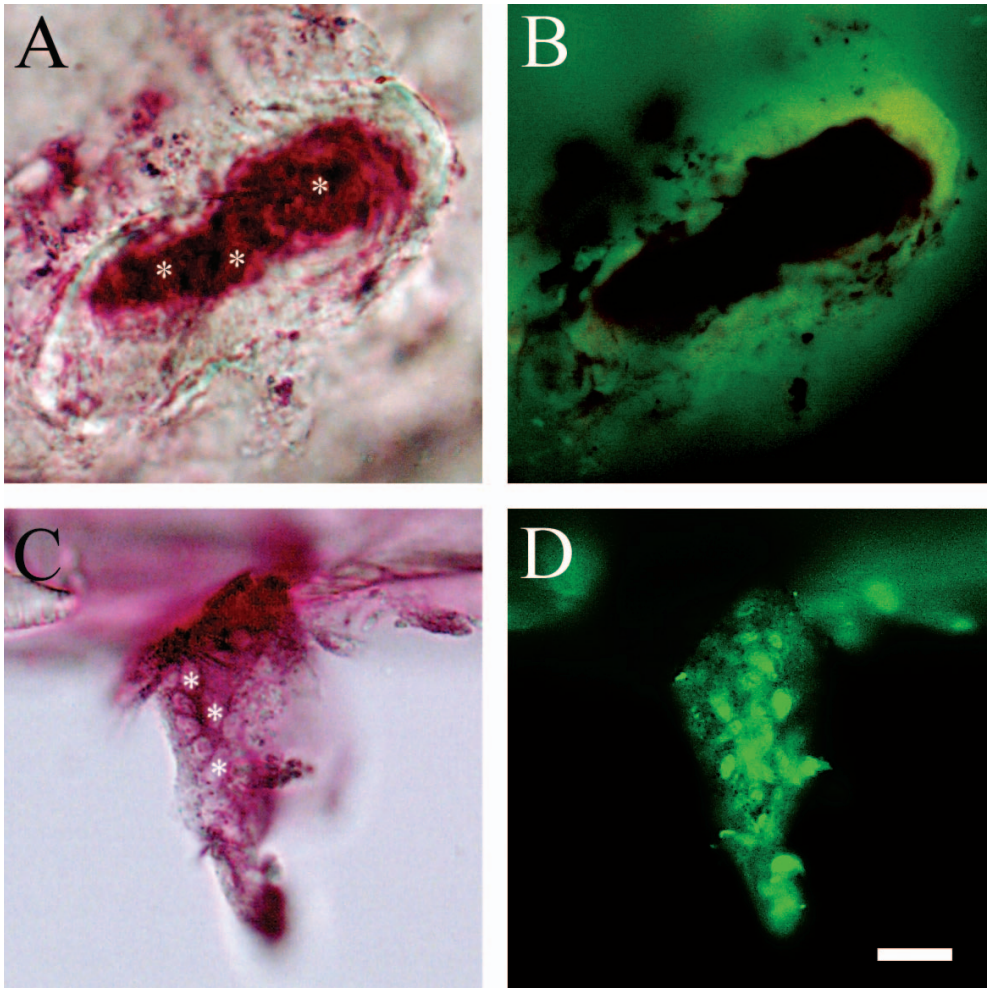

Fig. 3. Stained whole-mounted goldfish scales showing apoptosis labeled by TUNEL (fluorescence; **B, D**) of a TRAP-positive osteoclast (reddish-stained cell; **A, C**). TUNEL-positive multinuclei are evident in the osteoclast adjacent to the surface of a scale treated with PRL **(C, D)**, whereas the osteoclast is not labeled by TUNEL in the control scale **(A, B)**. Representative results are shown; no significant effect of PRL treatment was detected in the proportion of apoptotic osteoclasts compared with that seen in controls. Asterisks indicate the multinuclei typical of osteoclasts. Scale bar=20 μm.

hormone resulted in greater effects.

The multinuclei of some TRAP-stained osteoclasts in the scales were labeled with TUNEL to detect DNA breaks. No significant effect was seen on the proportion of these apoptotic osteoclasts by treatment with PRL (37±10%) compared with that seen in controls (30±9%; P=0.9; N=11) (Fig. 3).

Effects of PRL, GH and SL on osteoblastic activity in the scales

Fig. 4 shows the effects of PRL, GH, and SL on ALP activity as an osteoblastic marker. All the hormones promoted the activity by ~30%; SL was potent also at a concentration of 10 ng/ml. Increased concentrations of each hormone resulted in greater effects.

DISCUSSION

In the present study, we demonstrated for the first time that PRL inhibits osteoclastic activities. Conversely, GH and SL increased TRAP activities, indicating the specificity of the suppressive action of PRL in the osteoclasts. Furthermore, this in-vitro effect of PRL on goldfish scales occurred at a very low concentration (10 pg/ml), which was within the range of plasma PRL concentrations (~0.1–10 ng/ml) in teleosts, including the goldfish (Wong et al., 2002). Such a direct and specific effect of PRL has not been demonstrated before in teleosts, although PRL has been recognized to act on various tissues (see Sakamoto et al., 2003). Recently, some effects of PRL on cultured gill epithelia were reported, but the specificities of these effects were unclear (Kelly and Wood, 2003; Zhou et al., 2003, 2004). In-vivo sodium-retention bioassays, involving hypophysectomies and hormonal injections of fishes, have been used to test the bioactivity of PRLs in teleosts (Grau et al., 1984; Hasegawa et al., 1986; Suzuki et al., 1991; Jackson et al., 2000); however, except for transfection studies on PRL receptor cDNAs, our scale TRAP assay is the only in-vitro bioassay specific for teleost PRLs, one that can be completed in several hours.

The inhibitory effect of PRL on osteoclastic activity seems to be mediated in part through osteoclast apoptosis,

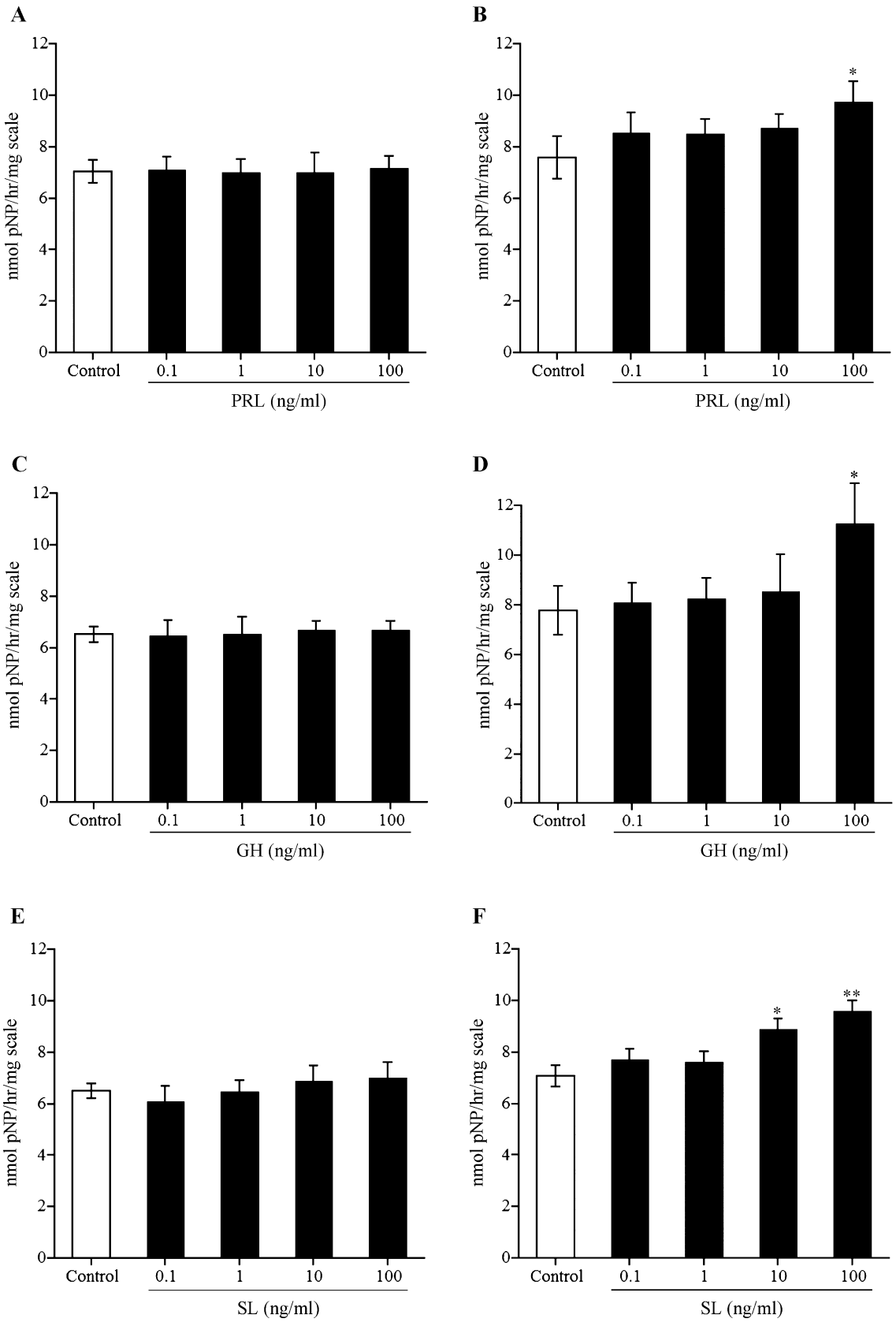

Fig. 4. Effects of PRL, GH, and SL (0.1 to 100 ng/ml) on ALP activity in cultured goldfish scales after 6 h **(A, C, E)** and 18 h **(B, D, F)** of incubation. Values are mean±SEM (N=8). * and ** indicate statistically significant differences at $P<0.05$ and 0.01, respectively, from the values in the control scales (ANOVA with Dunnett's post-hoc test).

since we observed apoptotic nuclei in TRAP-stained osteoclasts after PRL treatment. However, no statistically significant induction was detected, possibly due to the loss of TRAP activity at advanced apoptotic stages. Although further morphological characterization by transmission electron microscopy appears to be necessary, PRL was also shown to stimulate apoptosis in newt spermatogonia and rat luteal tissues (Kiya et al., 1998; Abe, 2004). Indeed, a large number of the effects of PRL reported throughout the vertebrates are directly associated with apoptosis and/or cell proliferation (Sakamoto and McCormick, 2006). The mechanisms by which PRL induces apoptosis are presently unclear and offer fertile ground for further investigation.

In the case of osteoblastic activities, significant stimulating effects of PRL, GH, and SL were observed at a concentration of 100 ng/ml, whereas SL increased the activities also at 10 ng/ml. GH can bind the receptor for SL, which plays a role in calcium metabolism (Kaneko, 1996), albeit with an 8-fold lower affinity than SL (Fukada et al., 2005). The osteoblastic activities of GH (and PRL) might be mediated through the SL receptors. Altogether, these inductions, as well as those of ALP, by GH and SL might be nonspecific/general actions of these cytokine hormones, whereas the inhibition of TRAP activity by PRL should be unique.

Our in-vitro study has demonstrated the first direct effects of PRL on scale/bone metabolism in a mature female fish. The contribution of these relatively modest changes in scale/bone metabolism to plasma calcium homeostasis appears to be counterintuitive and minor, since PRL is known to induce "hyper" calcemia in teleosts in vivo, mainly via the gill, even in fresh water, where calcium availability is limited (Wendelaar Bonga, 1997). On the other hand, as we observed for calcitonin (Suzuki et al., 2000), PRL may inhibit the excess degradation of bone tissue by osteoclasts in female goldfish, since plasma PRL levels usually increase during the reproductive period (see Sakamoto et al., 2003). During the reproductive period, estrogen stimulates bone degradation for the synthesis of vitellogenin, a Ca-binding protein (Suzuki and Hattori, 2002; Suzuki et al., 2004). Thus, PRL may act on calcium deposition into scales/bone independently of calcium homeostasis in teleosts.

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