Signal transduction mechanisms for autocrine/paracrine regulation of somatolactin-α secretion and synthesis in carp pituitary cells by somatolactin-α and -β

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Submitted 12 September 2012; accepted in final form 20 November 2012

Jiang Q, Wong AO. Signal transduction mechanisms for autocrine/paracrine regulation of somatolactin-α secretion and synthesis in carp pituitary cells by somatolactin-α and -β. Am J Physiol Endocrinol Metab 304: E176–E186, 2013. First published November 27, 2012; doi:10.1152/ajpendo.00455.2012.—Pituitary hormones can act locally via autocrine/paracrine mechanisms to modulate pituitary functions, which represents an interesting aspect of pituitary regulation other than the traditional hypothalamic input and feedback signals from the periphery. Somatolactin, a member of the growth hormone (GH)/prolactin (PL) family, is a pleiotropic hormone with diverse functions, but its pituitary actions are still unknown. Recently, two SL isoforms, SLα and SLβ, have been cloned in grass carp. Based on the sequences obtained, recombinant proteins of carp SLα and SLβ with similar bioactivity in inducing pigment aggregation in carp melanophores were produced. In carp pituitary cells, SLα secretion and cell content were elevated by static incubation with recombinant carp SLα and SLβ, respectively. These stimulatory actions occurred with a parallel rise in SLα mRNA level with no changes in SLβ secretion, cell content, and gene expression. In contrast, SLα mRNA expression could be reduced by removing endogenous SLα and immunoneutralization. At the pituitary cell level, SLα release, cell content, and mRNA expression induced by carp SLα and SLβ could be blocked by inhibiting JAK2/STAT5, PI3K/Akt, MEK1/2, and p38 MAPK, respectively. Furthermore, SLα and SLβ induction also triggered rapid phosphorylation of STAT5, Akt, MEK1/2, ERK1/2, MKK3/6, and p38 MAPK. These results suggest that 1) SLα and SLβ produced locally in the carp pituitary can serve as novel autocrine/paracrine stimulators for SLα secretion and synthesis and 2) SLα production induced by local release of SLα and SLβ probably are mediated by the JAK2/STAT5, PI3K/Akt, and MAPK signaling pathways.

SOMATOLACTIN, a member of the growth hormone (GH)/prolactin (PRL) family, is originally identified in the pars intermedia of the Atlantic cod (30). Based on comparative synteny and sequence homology analysis, it is commonly accepted that SL is derived from the ancestral GH during the early phase of gnathostome evolution by gene duplication (20). Two SL isoforms, SLα and SLβ, have been reported in bony fish (16, 52). Similar to GH and PRL, SL is a member of the class I cytokines with the typical feature of four α-helices arranged in an up-up-down-down topology (16). To date, SL has been associated with diverse functions in fish models, including reproduction (29, 36), stress (21), lipid metabolism (40), background adaptation (53, 55), phosphate transport (25), melanosome aggregation (28, 54), and immune responses (4). Although most of the above-mentioned functions attributed to SL were based on correlational studies, the functional role of SL in color presentation has been unequivocally demonstrated in fish species. Direct treatment with recombinant SL consistently induced melanosome aggregation in the melanophores of red drum (54) and zebrafish (28). Furthermore, disabling mutations (45) and/or genetic deletions (12) of SL have a common phenotype with a general loss of body pigment, suggesting that SL is also involved in chromatophore proliferation and differentiation.

To our knowledge, little is known regarding neuroendocrine regulation of SL production at the pituitary level. Based on the limited studies available in fish species, e.g., in the organ-cultured pituitary of rainbow trout, SL release was known to be upregulated by corticotropin-releasing factor (CRF) and gonadotropin-releasing hormone (GnRH) but suppressed by dopamine and serotonin (19). In a single study using primary culture of sea bass pituitary cells, high doses of leptin could induce SL release, whereas GnRH treatment had no effects in this regard (33). In grass carp pituitary cells, pituitary adrenocorticotropin (ACTH), cyclosporine (CAP), IL-1, and IGF-II have been identified to be potent stimulators for SL release, synthesis, and gene expression (16, 17). These findings suggest that SL gene expression and secretion are controlled by hypothalamic regulation as well as feedback signals from the periphery. Recently, a report on in vitro culture of cichlid fish pituitary has shown that treatment with exogenous LH and FSH could directly elevate SL secretion at the pituitary level (32), indicating that SL release is also under the control of autocrine/paracrine regulation of pituitary hormones.

In grass carp (Ctenopharyngodon idellus), GH autoregulation has been reported (50), which constitutes the signal amplification step of a novel intrapituitary feedback loop regulation GH gene expression via local interactions of gonadotrophs and somatotrophs (49). Given that 1) SL is derived from the ancestral GH gene by genome duplication (20) and 2) the localization of SLα- and SLβ-producing cells is a mosaic pattern in the pars intermedia of grass carp (17), we postulate that SL may retain the intrapituitary regulatory properties of GH to modulate SL secretion and synthesis via autocrine/paracrine mechanisms. To test the hypothesis, recombinant proteins for the two isoforms of grass carp SL, SLα and SLβ, were produced and confirmed to have comparable bioactivity in stimulating pigment aggregation in carp melanophores. By use of primary cultures of grass carp pituitary cells as a model, the pituitary actions of SL and post-receptor signaling mechanisms for SLα and SLβ regulation of SLα secretion and gene expression were investigated. In this study, we demonstrate for the first time that SL isoforms can act at the pituitary level to regulate SLα release and synthesis in autocrine/paracrine manners through JAK2/STAT5, PI3K/Akt and MAPK cascades.

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First published November 27, 2012; doi:10.1152/ajpendo.00455.2012.
MATERIALS AND METHODS

Animals. One-year-old grass carp with body weights of 1.5 to 2.0 kg were purchased from local wholesale markets and maintained in well-aerated 200-liter aquarium at 18°C under a 12:12-h light-dark photoperiod. Pituitary glands were obtained from prepubertal fish with no distinct sexual dimorphism. During the procedure of pituitary cell preparation, fish were euthanized by anesthesia in 0.05% MS222 (Sigma, St. Louis, MO) followed by spinosadectomy according to the protocol approved by the Committee of Animal Use at the University of Hong Kong (Hong Kong).

Test substances. Ovine PRL, porcine GH, α-melanocyte-stimulating hormone (α-MSH), and norepinephrine (NE) were purchased from Sigma (St. Louis, MO). Pharmacological agents, including wortmannin, LY-294002, IL-6-6-hydroxymethyl-chiro-inositol-2-R-2-O-methyl-3-O-octadecylcarbonyl (HIMOC), 5-dihydro-5-methyl-1-β-ribofuranosyl-1,4,5,6-tetraazaacenaphthlen-3-amine (API-2), PD-98059, U-0126, SB-203580, PD-1693161, AG-490 and N-(4-oxo-4H-chromen-3-yl)acetylhydrazide (Nico) were obtained from Calbiochem (San Diego, CA). PRL, GH, α-MSH, and NE were prepared as a 1 mM stock solutions in small aliquots frozen at −80°C. Similar to peptide hormones, the pharmacological agents targeting different signaling pathways were prepared as 10 mM frozen stocks except that they were dissolved in dimethyl sulfoxide (DMSO) as the solvent. On the day of experiment, stock solutions of test substances were diluted with prewarmed (28°C) culture medium to appropriate concentrations 15 min prior to drug treatment. In these experiments, the final dilutions of DMSO were always less than 0.1% and did not affect SL secretion, protein production, and transcript expression in grass carp pituitary cells.

Recombinant protein for grass carp SLα and SLβ. In our previous study (16), the full-length cDNA clones coding for grass carp SLα (GenBank acc. no. EF372074) and SLβ (GenBank acc. no. EF372075) were isolated from the carp pituitary. The open reading frame (ORF) of the respective SL isoforms were PCR isolated using an Expand High Fidelity system using primers specific for grass carp SLα [forward primer: 5'-TATAGCATCCATGAACAGGGTTAAAGTTCTG-3' (BamHI site underlined) and reverse primer: 5'-TATACCTCGAGTTAGTGGGAAAGACAACTGAG-3' (XhoI site underlined)] and SLβ [forward primer: 5'-TCTCGAATTCCATGAGAACGAAATACGAGTTCTCT-3' (EcoRI site underlined) and reverse primer: 5'-TATACCTCGAGTTAGAAAAGAGGAGACATCTTTC-3' (XhoI site underlined)], respectively. The restriction sites introduced into the respective primers were used for subsequent cloning of PCR products into the prokaryotic expression vector pET28a His-tag vector (Novagen, Madison, WI). PCR amplification of SLα and SLβ was initiated by denaturation at 94°C for 1 min followed by primer annealing at 55°C for 1 min and extension at 72°C for 2 min for a total of 25 cycles. After size fractionation by 1% agarose gel electrophoresis, 708 bp and 687 bp PCR products (for SLα and SLβ ORF, respectively) were gel purified using a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and subcloned into the prokaryotic expression vector pET28a His-tag vector. Sequence identity of the SLα and SLβ inserts was confirmed by DNA sequencing using an ABI PRISM dRhodamine dye Terminator Cycle Sequencing kit (PerkinElmer, Foster City, CA). The expression vectors generated, namely “pET28a-SLα” and “pET28a-SLβ”, were introduced into E. coli BL21(DE3)-competent cells by “heat shock” transformation, and single colonies of transformed bacteria were used for recombinant protein production in 250 ml of Luria-Bertani (LB) medium with 50 μg/ml antibiotic ampicillin at 28°C with constant shaking (220 rpm). IPTG induction (0.5 mM, Sigma-Aldrich, St. Louis, MO) was added when the OD600 reading of bacteria culture reached the level of 0.4–0.5. After incubation for appropriate duration, the bacteria with SL protein expression were harvested by centrifugation at 3,000 g for 20 min at 4°C and resuspended in 1× PBS (pH 8.0) for protein preparation. In this case, the cell suspension obtained was then subjected to three cycles of repeated freezing and thawing followed by a 5-min sonication using a Branson Sonifier 250 (VWR Scientific, San Diego, CA). Maximal preparations of soluble protein fractions (~30%) can be reached after IPTG (0.5 mM) induction at the optimal time point for protein expression (12 h). Insoluble and insoluble protein fractions of the cell lysate were isolated by high-speed centrifugation at 12,000 g for 20 min at 4°C. After that, grass carp SL expressed as His-tagged recombinant protein were purified from soluble fraction by immobilized metal ion affinity chromatography using Ni-NTA Superflow columns (Qiagen) according to the manufacturer’s instructions. Determining the identity of recombinant proteins was confirmed by Western blot of anti-6 × His-tag antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-SLα (1:2,000,000), and SLß (1:1,000,000) antisem, respectively. The recombinant proteins produced were quantified using a BCA protein assay kit (Pierce, Rockford, IL) and tested for bioactivity using a pigment aggregation assay in grass carp melanophores.

Pigment aggregation assay in carp melanophores. To establish a pigment aggregation assay for the testing of SL bioactivity, scales with abundant levels of epidermal melanophores were collected from the base of the dorsal fin of individual grass carp. The scales collected were washed three to five times using fish physiological saline (pH 7.4, in g/liter: 6.42 NaCl, 0.15 KCl, 0.22 CaCl2, 0.12 MgSO4, 0.084 NaHCO3, and 0.06 NaH2PO4) to remove the mucus and then cultured in six-well plates (Corning-Costar, Oneonta, NY) with MEM medium (pH 7.5; Invitrogen, Carlsbad, CA) at 28°C under 5% CO2 and saturated humidity. The culture medium was changed daily to remove the mucus secreted on the surface of the scale. On the fourth day, old culture medium was replaced with MEM containing the recombinant protein of grass carp SLα and SLβ. After incubation with drug treatment for 2 h, the scale of epidermal melanophores with different levels of pigment aggregation was evaluated by cell counting under an inverted microscope (Leica, Solms, Germany), and the extent of pigment dispersion was scored as aggregated, partially dispersed, or fully dispersed, according to the melanophore index (M.I.) described by Hogben et al. (13). Given that NE could induce melanosome aggregation in fish scales (28, 54), parallel treatment with NE was used as a positive control for pigment aggregation in melanophores located within the scale epithelia.

SL mRNA measurement in carp pituitary cells. Grass carp pituitary cells were prepared by trypsin-DNase digestion method as described previously (15). After endotoxin digestion, pituitary cells were seeded in 24-well clustered plates at a density of 2.5 × 106 cells per well and cultured overnight at 28°C in carp MEM (MEM edium supplemented with 26 mM NaHCO3, 25 mM HEPES, 100 U/ml penicillin, 100 g/ml streptomycin, and 250 ng/ml fungizone, pH 7.7) with 5% FBS. After overnight recovery, static incubation with test substances was conducted for 48 h based on our time course studies (data not shown). After that, total RNA was isolated and reverse transcribed using Superscript II (50 Unit, Invitrogen). The RT samples obtained were subjected to real-time PCR using a LightCycler SYBR Green I Kit (Roche, Stockholm, Sweden) with primers specific for grass carp SLα (forward primer 5'-ACCCACTGTTACTCTCAAATCCTC-3', reverse primer 5'-CTCTGAACGATCAAGAGTAGT-3'), and SLβ (forward primer 5'-TGTTT GAGGAGATGCTCGTTT-3', reverse primer 5'-CCACGTCACCAATATCTGTG-3'). Real-time PCR for the two SL targets was performed with initial denaturation at 94°C for 3 min followed by 35 cycles of amplification with denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s. These primers consistently produced a single PCR product of 304 bp (for SLα) and 310 bp (for SLβ) for SLα and SLβ mRNA, respectively. Based on our validation, the primers for SLα did not “cross-amplify” SLβ mRNA and vice versa. Serial dilutions of plasmid DNA carrying full-length SLα and SLβ cDNA were used as the standards for data calibration, and parallel real-time PCR for β-actin was used as the internal control.

Western blotting. The neuromedin lobe (NIL) of the carp pituitary was manually dissected under a stereomicroscope and used for preparation of pituitary cells enriched with SL cell populations.
RESULTS

Production of recombinant grass carp SLα and SLβ. To produce recombinant proteins for grass carp SLα and SLβ, *E. coli* BL21 (DE3) cells were transformed with pET28a-SLα and pET28a-SLβ, respectively. After IPTG induction, recombinant protein expression (31 kDa for SLα and 30 kDa for SLβ) became noticeable at 6 h and maintained at high levels up to 12 h (Fig. 1A). Therefore, large-scale protein expression was routinely conducted in BL21 (DE3) cells with 12-h IPTG induction at 28°C. As shown in Fig. 1B, recombinant proteins of appropriate size for SLα and SLβ were not apparent in bacterial cells transformed with the blank vector or without IPTG induction. After IPTG induction, target protein of 31 kDa for SLα and 30 kDa for SLβ were detected in both soluble and insoluble cell lysates. These protein bands could also be recognized by the antisera for 6 × His-tag (1:1,000; Santa Cruz Biotechnology), confirming that they are the 6 × His-tag
fusion protein of the respective isoforms of grass carp SL. In the following purification step, only the soluble fraction was used for protein purification by immobilized metal ion affinity chromatography. The 6 x His fusion protein of SLα and SLβ in the soluble fraction of bacterial lysates was trapped with Ni-NTA columns, and contaminating proteins of bacterial origin were removed by repeated washing. As revealed by Coomassie blue staining, the final products of SLα and SLβ eluted from the Ni-NTA columns were at high-quality purification without noticeable levels of contaminating proteins. Based on densitometry scanning of the target band, the overall yield of purified SLα and SLβ amounted to 95% and 94% at the final purification stage, respectively.

**Bioactivity of recombinant SLα and SLβ.** To test the bioactivity of recombinant SLα and SLβ, an in vitro culture system of grass carp scales was established. The dorsal scales of grass carp contain a high level of melanophores in the epithelial layer, which represents an ideal system to test the functionality of grass carp SLα and SLβ. Based on our validation using the melanophore index to evaluate the extent of pigment dispersion (Fig. 2A), 2-h static incubation of recombinant proteins of grass carp SLα (1 µM) and SLβ (1 µM) was found to be effective in triggering maximal pigment aggregation (level 1 pigment dispersion) in more than 80% of the melanophores examined (Fig. 2B). To evaluate the relative potency of the two SL isoforms, dose-response studies were also performed with the recombinant protein of grass carp SLα and SLβ. As shown in Fig. 2C, both isoforms of carp SL were effective in increasing pigment aggregation in a concentration-related fashion, with comparable ED50 values (ED50 for SLα 7.5 ± 1.5 nM and for SLβ 9.8 ± 2.4 nM). Given that NE is known to act directly on fish melanophores to induce pigment aggregation (28, 54), parallel treatment with NE (1 µM) was used as a positive control in these experiments (Fig. 2, B and C). Similar to the results of SLα and SLβ, NE treatment consistently induced pigment aggregation in most of the surface melanophores.

**SL-induced SLα production and mRNA expression.** To test the pituitary actions of SL in a fish model, static incubation of grass carp pituitary cells was performed with increasing concentration (0.1–100 nM) of recombinant carp SLα and SLβ, respectively. As revealed by the results of Western blotting, basal levels of SLα secretion, cell content, and total production were elevated in a dose-dependent manner by grass carp SLα and SLβ (Fig. 3, A and B). Parallel blotting for SLβ in these protein samples, however, did not reveal significant changes in SLβ secretion, cell content, and total production (data not shown). Consistent with the rises in SLα cell content and total production, SLα and SLβ treatment were also effective in elevating SLα mRNA levels in a dose-dependent manner (Fig. 3, C and 3D). Again, parallel measurement of SLβ mRNA in the same samples revealed no significant change in basal levels of SLβ transcript. To confirm the specificity of SL action, the effects of other pituitary hormones, including GH, PRL, and α-MSH were also tested. Since 1) the amino acid sequences of carp α-MSH were identical to mammalian α-MSH (2) and 2) mammalian GH and PRL are also biologically active in fish models (5, 14, 37, 50), porcine GH, ovine PRL, and human α-MSH were used in our present studies. In this case, static incubation with PRL (100 nM) and α-MSH (100 nM) did not alter basal expression of SLα and SLβ mRNA; however, GH (100 nM) treatment slightly inhibited SLα gene expression but had no effect on SLβ mRNA expression in carp pituitary cells (Fig. 4A). Furthermore, cotreatment with SLα and SLβ did not further enhance the stimulatory action of individual isoforms on SLα mRNA expression (Fig. 4B), suggesting that the effects of SLα and SLβ on SLα mRNA expression are not additive. To further evaluate the functional role of SL produced locally

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at the pituitary level in SLα gene regulation, immunoneutralization was performed in grass carp pituitary cells with specific antiserum for grass carp SLα and SLβ, respectively. The specificity of antiserum against carp SLα and SLβ was confirmed by our recent findings (17). In these experiments, removal of endogenous SLα and SLβ secreted into the culture medium by the respective antiserum was effective in suppressing basal SLα mRNA level in a dose-dependent manner (Fig. 4, C and D). On the other hand, treatment with the respective preimmunized serum (1:2,500, PS) for SLα and SLβ had no significant effects on SLα gene expression.

**Signal transduction for SL-induced SLα expression.** Given that SL receptor is considered to be a paralogue of type I GH receptor in fish model (11, 18, 34), the possible involvement of the JAK2/STAT5 pathway for SL induction of SLα secretion and synthesis was tested. In grass carp pituitary cells, increasing concentrations (0.1–100 nM) of SLα and SLβ were both effective in triggering STAT5 phosphorylation in a dose-dependent manner without significant changes in total STAT5 content at the cell level (Fig. 5, A and B). In parallel experiments, the stimulatory effects of SLα and SLβ on SLα release, cell content, and mRNA expression were inhibited by simultaneous treatment with the JAK2 inhibitor AG-490 (100 μM) and STAT5 inhibitor Nico (50 μM) (Fig. 5, C and D). Since JAK2 activation can also activate PI3K- and MAPK-dependent pathways coupled to GH receptor (35), the functional role of these two signaling cascades in SLα expression was also investigated. Consistent with this hypothesis, the levels of phosphorylated Akt were increased in a concentration-related fashion without major changes in total protein of Akt (Fig. 6, A and B). Also, Akt phosphorylation induced by SLα and SLβ stimulation could be negated by the PI3K inhibitor wortmannin (10 nM; Fig. 6, C and D). Furthermore, as shown in Fig. 6, E and F, SLα secretion, cell content, and mRNA expression induced by SLα and SLβ could be blocked by cotreatment with the PI3K inhibitor wortmannin (10 nM) and Akt inhibitor API-2 (10 μM), respectively. A similar inhibitory action was also observed by PI3K inhibitor LY-294002 (10 μM) and Akt inhibitor HIMOC (10 μM) (data not shown). In parallel experiments, SLα and SLβ treatment were both effective in triggering protein phosphorylation of the major components of the MAPK pathway, including MEK1/2 (Fig. 7, A and B) and MKK3/6 (Fig. 8, A and B). Besides, the MEK1/2 inhibitor PD-98059 (10 μM) and p38 MAPK inhibitor SB-203580 (10 μM) were both effective in altering SLα and SLβ expression. Effects of SLα (10 nM) and SLβ (10 nM) alone or together on SLα mRNA expression. Effects of SLα (C) and SLβ (D) immunoneutralization on SLα mRNA expression in grass carp pituitary cells. Pituitary cells were exposed to increasing levels of SLα and SLβ antiserum for 48 h. In this case, treatment with preimmunized serum (PS; 1:2,500,000) was not effective in altering SLα and SLβ mRNA expression. After drug treatment, SLα mRNA expression was measured by real-time PCR. Data presented for SL mRNA are expressed as means ± SE (n = 4), and groups denoted by the same letter represent a similar magnitude of SL gene expression (P < 0.05, ANOVA followed by Fisher’s LSD test).
followed by Fisher’s LSD test). 

...mRNA expression, pituitary cells were treated with SLa and SLβ-induced Akt activation. E: effects of PI3K/Akt inhibition on SLα- and SLβ-induced SLα secretion, cell content, and mRNA expression. In these experiments, NIL cells prepared from carp pituitary with increasing doses SLα or SLβ (0.1–100 nM) for 48 h with cotreatment of PI3K inhibitor wortmannin (10 nM). After drug treatment, Akt phosphorylation was monitored by Western blot (gel bands). Blots represent prototypical examples of experiments replicated at ≥3 times. Western blotting analysis was performed as described in MATERIALS AND METHODS (bar graph). In parallel experiments for SL secretion, cell content, and mRNA expression, pituitary cells were treated with SLα and SLβ (10 nM) for 48 h with cotreatment of JAK2 inhibitor AG-490 (100 µM) and STAT5 inhibitor Nico (50 µM). After that, SLα immunoreactivities were monitored by Western blot using antiserum for SLα (1:2,000,000). Parallel blotting of β-actin was used as internal control. Total RNA was isolated for SLα mRNA measurement using real-time PCR (bar graph). Data presented for SL mRNA are expressed as means ± SE (n = 4), and groups denoted by the same letter represent a similar magnitude of SL gene expression (P < 0.05. ANOVA followed by Fisher’s LSD test).

μM) also confirmed that these inhibitors were effective in blocking SLα and SLβ induced ERK1/2 (Fig. 7, C and D) and p38 MAPK (Fig. 8, C and D) phosphorylation, respectively, in grass carp pituitary cells. Furthermore, SLα and SLβ induced SLα secretion, cell content and mRNA expression were found to be sensitive to the blockade by the MEK1/2 inhibitor PD-98059 (10 µM; Fig. 7, E and F) and p38 MAPK inhibitor SB-203580 (10 µM; Fig. 8, E and F). These observations are in agreement with parallel experimental results by using another MEK1/2 inhibitor, U-0126 (10 µM), and the p38 MAPK inhibitor PD-169816 (10 µM) (data not shown).

To establish the functional hierarchy of JAK2 with respect to PI3K- and MAPK-dependent pathways, SLα- and SLβ-induced ERK1/2, Akt, and p38 MAPK phosphorylation were tested in the presence of the JAK2 inhibitor AG-490 (100 µM). In these experiments, the phosphorylation response for ERK1/2 (Fig. 9, A and B) and Akt (Fig. 9, C and D) were markedly suppressed, whereas p38 MAPK phosphorylation (Fig. 9, E and F) induced by SLα and SLβ treatment was not affected by AG-490. Interestingly enough, SLα- and SLβ-induced ERK1/2 phosphorylation observed in carp pituitary cells could be abolished by cotreatment with the PI3K inhibitor wortmannin (10 nM; Fig. 9, A and B). In contrast, the corre-
expression. In addition, this cross-talk is also sensitive to another PI3K inhibitor, LY-294002 (10 µM), and MEK1/2 inhibitor U-0126 (10 µM) (data not shown).

**DISCUSSION**

Since 1) GH-induced GH secretion and synthesis via GHR activation has been reported in grass carp pituitary cells (50),

**Fig. 7.** Effects of MEK1/2 ablation on SLα and SLβ upregulation of SLα expression in grass carp pituitary cells. Dose dependence of SLα (A) and SLβ (B) induction on MEK1/2 phosphorylation. C and D: effects of MEK1/2 inhibition on SLα- and SLβ-induced ERK1/2 activation. E and F: effects of MEK1/2 inhibition on SLα- and SLβ-induced SLα secretion, cell content, and mRNA expression. In these experiments, NIL cells prepared from carp pituitary for 30 min with increasing doses SLα or SLβ (0.1–100 nM) or with cotreatment of MEK1/2 inhibitor PD-98059 (10 µM). After drug treatment, MEK1/2 and ERK1/2 phosphorylation was monitored by Western blot (gel bands). Blots represent prototypical examples of experiments replicated ≥3 times. Parallel blotting of total MEK1/2, ERK1/2, and β-actin was performed to serve as internal control. Western blotting analysis was performed as described in MATERIALS AND METHODS (bar graph). In parallel experiments for SLα secretion, cell content, and mRNA expression. Pituitary cells were treated for 48 h with 10 nM SLα and SLβ in the presence or absence of MEK1/2 inhibitor PD-98059 (10 µM). After drug treatment, SLα immunoreactivities were monitored by Western blot using antisera for SLα (1:2,000,000). Parallel blotting of β-actin was used as internal control (gel bands). Total RNA was isolated for SLα mRNA measurement using real-time PCR (bar graph). Data presented for SLα mRNA are expressed as means ± SE (n = 4), and groups denoted by the same letter represent a similar magnitude of SL gene expression (P < 0.05, ANOVA followed by Fisher’s LSD test).

Corresponding responses on Akt phosphorylation were sensitive to the blockade of the MEK1/2 inhibitor PD-98059 (10 µM; Fig. 9, C and D), suggesting that a functional cross-talk between the PI3K and MAPK cascades may play a role in SLα gene

**Fig. 8.** Functional role of p38 MAPK cascades in SLα and SLβ upregulation of SLα expression in grass carp pituitary cells. Dose dependence of SLα (A) and SLβ (B) induction on MKK3/6 phosphorylation. C and D: effects of p38 MAPK inhibition on SLα- and SLβ-induced p38 MAPK activation. E and F: effects of p38 MAPK inhibition on SLα- and SLβ-induced SLα secretion, cell content, and mRNA expression. In these experiments, NIL cells prepared from carp pituitary for 30 min with increasing doses SLα or SLβ (0.1–100 nM) or with cotreatment of p38 MAPK inhibitor SB-203580 (10 µM). After drug treatment, MKK3/6 and p38 MAPK phosphorylation was monitored by Western blot (gel bands). Parallel blotting of total MKK3/6, p38 MAPK, and β-actin was performed to serve as internal control. Blots represent prototypical examples of experiments replicated ≥3 times. Western blotting analysis was performed as described in MATERIALS AND METHODS (bar graph). In parallel experiments for SLα secretion, cell content, and mRNA expression, pituitary cells were treated with SLα and SLβ (10 nM) for 48 h with cotreatment of p38 MAPK inhibitors SB-203580 (10 µM). After drug treatment, SLα immunoreactivities were monitored by Western blot using antisera for SLα (1:2,000,000). Parallel blotting of β-actin was used as internal control (gel bands). Total RNA was isolated for SLα mRNA measurement using real-time PCR (bar graph). Data presented for SLα mRNA are expressed as means ± SEM (n = 4), and groups denoted by the same letter represent a similar magnitude of SL gene expression (P < 0.05, ANOVA followed by Fisher’s LSD test).
and 2) SL receptors with cross-reactivity for SL and GH binding in salmon (10) have been classified as a member of the GHR family in fish models (11); we suspect that SL regulation via local actions of SLα and SLβ may also exist in the carp pituitary. As a first step to test our hypothesis, recombinant proteins for grass carp SLα and SLβ were produced in E. coli and purified to almost homogeneity by immobilized metal ion affinity chromatography. The recombinant proteins ran at 31 kDa (for SLα) and 30 kDa (for SLβ) on SDS-PAGE, slightly larger than the predicted MWs of 30.1 and 28.9 kDa, respectively (including the His-tag). One possible explanation is that the basic residues of His-tag contain a high level of positive charges, resulting in a higher apparent MW (41). Another possible explanation is that the hydrophilic moieties in SLα and SLβ may obstruct the binding of SDS by decreasing the hydrophobic interaction between the protein and SDS (39), leading to a higher apparent MW by reducing the overall charges on the protein molecule. In our case, we could not rule out the possibility that other amino acid sequences in SLα and SLβ may also exist in the carp scale. In our case, we could not rule out the possibility that other amino acid sequences in SLα and SLβ may also exist in the carp scale.
In primary cultures of grass carp pituitary cells, the recombimant proteins for carp SLα and SLβ were both effective in elevating SLα secretion, SLα cell content, and total SLα protein production. Since the increase in SLα protein expression also occurred with a concurrent rise in SLα mRNA level, the stimulatory effects on SL production was probably the result of SL-induced SLα gene expression. In parallel studies, corresponding changes in SLβ protein levels and gene expression were not apparent after SL treatment. Furthermore, the stimulatory effects of SLα and SLβ on SLα mRNA expression were not additive and could not be mimicked by parallel treatment with GH, PRL (members of the same hormone families), and α-MSH (hormone produced by NIL cells in close proximity to SL cells). It is also worth mentioning that GH might act in a paracrine manner to inhibit SLα but not SLβ gene expression, which agrees with the recent results by Kurata et al. (22). More importantly, removal of endogenous SLα and SLβ secreted in pituitary cell culture by immunoneutralization with the respective antisera could dose-dependently suppress SLα transcript level. These results demonstrated for the first time that locally produced SLα and SLβ can serve as an autocrine/paracrine stimulator in the carp pituitary to upregulate SLα secretion and synthesis. These stimulatory effects are SL specific and not caused by cross-reactivity with GH and/or PRL receptors or indirect action of other pituitary hormone (e.g., α-MSH) expressed in the NIL. In the same study, a lack of additivity for the two SL isoforms on SLα gene expression was also observed. “Cross-desensitization” by SLα and SLβ treatment was suspected. In this case, SLα cells might have been desensitized by SLα stimulation and failed to respond to a second dose of SLβ.

At present, except for a single study in flounder renal tubule cells showing that the cAMP/PKA pathway is involved in SL-induced phosphate transport (25), the post-receptor for signaling mechanisms coupled with SL receptors are largely unknown. In mammals, GH binding to preimerized GHR via site 1 and site 2 interactions (46) leads to JAK2 recruitment and subsequent activation of STAT, MAPK, and IRS/Pi3K pathways via the assembly of a multiple-protein signaling complex (6, 8). Given that 1) SL receptor has been classified as a member of the GHR1 family (11) and 2) coevolution of structures and functions of homologous ligands and their receptors are known to be a common phenomenon in vertebrate species, e.g., gonadotropins and their receptors (27) and ligand-receptor pairs of vasopressin/oxytocin superfamily (42), the possible involvement of these signaling cascades in SL-induced SLα release and synthesis was also examined. In grass carp pituitary cells, SLα and SLβ induction could trigger phosphorylation of STAT5, Akt, MEK1/2, ERK1/2, MKK3/6, and p38 MAPK. Furthermore, the stimulatory effects on SLα secretion, cell content, and mRNA expression induced by SLα and SLβ were totally inhibited by the JAK2 inhibitor AG-490, STAT5 blocker Nico, PI3K inhibitor wortmannin, Akt inhibitor API-2, MEK1/2 inhibitor PD-98059, and p38 MAPK inhibitor SB-203580. These results, as a whole, provide evidence that SL stimulation at the pituitary level could activate the JAK2/STAT5, P38/PI3K/Akt, MEK/ERK1/2, and MKK3/6/p38 MAPK cascades, which may contribute to SL induction of SLα secretion and synthesis in the carp pituitary.

In mammalian cell models, JAK2 activation can also recruit various members of the MAPK cascades, including ERK1/2, p38 MAPK, and JNK via the Grb2/SOS scaffold complex (3). In primary adipocyte culture, GH receptor activation can induce IRS-1 phosphorylation and recruitment of PI3K (23), presumably via FAK/Pyk2 association with JAK2 (51). In our study with primary culture of carp pituitary cells, phosphorylation of ERK1/2 and Akt, but not p38 MAPK, induced by SLα and SLβ treatment could be blocked by the JAK2 inhibitor AG-490, suggesting that the MEK/ERK1/2 and PI3K/Akt pathways are acting downstream of JAK2 activation. Besides JAK2 inactivation, SL-induced ERK1/2 phosphorylation in carp pituitary cells could also be suppressed by the PI3K inhibitor wortmannin. In reciprocal experiments, SL-induced Akt phosphorylation was also negated by cotreatment with the MEK1/2 inhibitor PD-98059. These results imply that a functional cross-talk between PI3K/Akt and MEK/ERK1/2 cascades may be involved in SL induction of SLα gene expression in the carp pituitary. Functional cross-talk between PI3K/Akt and MEK/ERK1/2 pathways is well documented and represents a major area in cancer research and drug design (1, 7). In mammalian cell models, the PI3K/Akt pathway can impact

Fig. 10. A: schematic diagram of autocrine/paracrine regulation of SLα expression by SLα and SLβ in grass carp pituitary cells. B: summarized diagram of the signal transduction pathways of SLα- and SLβ-mediated SLα expression in grass carp pituitary cells.
different actions on MAPK cascades being stimulatory via PI3K activation of Ras, which can lead to ERK1/2 activation (47) or inhibition via Akt phosphorylation of Raf (e.g., B-Raf and C-Raf) to reduce its stimulatory effect on the MEK/ERK1/2 pathway (56). In colon cancer cell lines (e.g., HT29 and Caco-2 cells), the downstream effector of Akt, namely GSK3 (glycogen synthase kinase-3), is also known to inhibit ERK1/2 phosphorylation, and this inhibitory action is mediated by PKC\(\beta\) activation (44). Regarding the reciprocal modulation of the PI3K/Akt pathway by MAPK, Ras, the upstream activator of the MEK/ERK1/2 pathway, has been shown to facilitate PI3K anchoring to the plasma membrane, which is essential for the full activation of the lipid kinase (9). In NIH 3T3 and MCF-7 cells, the PI3K/Akt pathway can be upregulated by a constitutively active mutant of Ras through inhibition of expression via MEK/ERK1/2 cascades (43). Furthermore, ERK1/2 activation can also induce TSC2 (tuberous sclerosis 2) phosphorylation to enhance mTORC1 (mammalian target of rapamycin complex 1) signaling, which is a major downstream target of the PI3K/Akt pathways (26), suggesting a functional convergence between the two pathways.

In summary, recombinant protein of grass carp SL\(\alpha\) and SL\(\beta\) with comparable bioactivity in inducing pigment aggregation in carp melanophores have been produced. Using these recombinant proteins, together with the specific antiserum for SL\(\alpha\) and SL\(\beta\) respectively, we have demonstrated for the first time that locally produced SL\(\alpha\) and SL\(\beta\) can act in an autocrine/paracrine manner to stimulate SL\(\alpha\) secretion and synthesis in the carp pituitary (Fig. 10A). The rise in SL\(\alpha\) protein production was contributed to be the result of SL\(\alpha\) gene expression triggered by SL activation of JAK2/PI3K/Akt, JAK2/MEK/ERK1/2, and MKC\&/p38 MAPK pathways (Fig. 10B). In our present study, SL could not be effective in elevating cAMP release and total production at the pituitary level (data not shown), suggesting that SL may selectively assemble different signaling complexes in a tissue-specific manner. However, it remains to be determined whether JAK2 is also coupled with the c-Jun NH\(\_\)terminal protein kinase (JNK) pathway to control SL expression at the pituitary level. Also, since pharmacological inhibitors might have side effects (especially at high doses), which may limit the interpretation of experimental results, future studies are required to further examine how these signals contribute to biological roles of somatolactin by using pharmacological inhibitors in combination with dominant-negative mutants for the respective signaling targets. Given that two SL isoforms, SL\(\alpha\) and SL\(\beta\), have the low amino acid similarities along with different expression patterns in the pituitary and periphery (16), further investigation is clearly warranted to elucidate the overlapping yet distinct functions of SL\(\alpha\) and SL\(\beta\) in fish.

ACKNOWLEDGMENTS

We gratefully acknowledge Wendy Ko for skillful technical assistance. We also thank the anonymous reviewers for their constructive suggestions to improve this manuscript.

GRANTS

GRF grants (RGC, HK) and National Basic Research Program of China (2010CB126302) to A.O.L., Wong and Small Project Funding (HKU, HK) to Q. Jiang Financial support from the School of Biological Sciences (University of Hong Kong) in the form of a postdoctoral fellowship (to Q. Jiang) is also acknowledged.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Q.J. and O.W. conception and design of research; Q.J. performed experiments; Q.J. and O.W. analyzed data; Q.J. and O.W. interpreted results of experiments; Q.J. and O.W. prepared figures; Q.J. drafted manuscript; Q.J. and O.W. edited and revised manuscript; Q.J. and O.W. approved final version of manuscript.

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