Somatostatin inhibits hepatic growth hormone receptor and insulin-like growth factor I mRNA expression by activating the ERK and PI3K signaling pathways

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Hagemeister AL, Sheridan MA. Somatostatin inhibits hepatic growth hormone receptor and insulin-like growth factor I mRNA expression by activating the ERK and PI3K signaling pathways. Am J Physiol Regul Integr Comp Physiol 295: R490–R497, 2008. First published May 21, 2008, doi:10.1152/ajpregu.00099.2008.—Previsously, we reported that somatostatins (SS) inhibit organismal growth by reducing hepatic growth hormone (GH) sensitivity and by inhibiting insulin-like growth factor I (IGF-I) production. In this study, we used hepatocytes isolated from rainbow trout to elucidate the mechanism(s) associated with the extrapituitary growth-inhibiting actions of SS. SS-14, a predominant SS isoform, stimulated tyrosine phosphorylation of several endogenous proteins, including extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family, and protein kinase B (Akt), a downstream target of phosphatidylinositol 3-kinase (PI3K). SS-14 specifically stimulated the phosphorylation of both ERK 1/2 and Akt in a concentration-dependent fashion. This activation occurred within 5–15 min, then subsided after 1 h. The ERK inhibitor U0126 retarded SS-14-stimulated phosphorylation of ERK 1/2, whereas the PI3K inhibitor LY294002 blocked SS-14-stimulated phosphorylation of Akt. SS-14-inhibited expression of GH receptor (GHR) mRNA was blocked by U0126 but not by LY294002. By contrast, U1026 had no effect on SS-14 inhibition of GH-stimulated IGF-I mRNA expression, whereas LY294002 partially blocked the inhibition of GH-stimulated IGF-I mRNA expression by SS-14. These results indicate that SS-14-inhibited GH mRNA expression is mediated by the ERK signaling pathway and that the PI3K/Akt pathway mediates, at least in part, SS-14 inhibition of GH-stimulated IGF-I expression.

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In this study, we used hepatocytes isolated from rainbow trout to elucidate the signaling pathways that underlie the extrapituitary actions of SS on growth. Specifically, we tested the hypothesis that SS-inhibited GHR and IGF-I expression is mediated by the extracellular signal-regulated (ERK) family of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways. Rainbow trout were used because their SS and GH-IGF-I systems are well characterized and display substantial structural diversity among their elements (e.g., multiple SS isoforms and receptor subtypes, multiple GHRs) systems (5). Our focus was on liver because it possesses the greatest number of GHRs and expresses more IGF-I than any other vertebrate tissue (3, 24, 30).

**METHODS**

**Materials.** All chemicals and reagents were purchased from Sigma (St. Louis, MO) unless noted otherwise. PPSS I-derived peptide hormones (SS-14-I, SS-26-I, and SS-28-I) were purchased from Sigma, whereas PPSS II-derived hormones (SS-14-II, SS-25-II, and SS-28-II) were custom synthesized by Dr. D. Smith. Trout GH was obtained from GroPep (Adelaide, Australia). Anti-phosphotyrosine rabbit antibody was purchased from Calbiochem (La Jolla, CA). Phospho-specific and control rabbit antibodies for human ERK 1/2 (phosphorylated at Thr202/Tyr204) and Akt (phosphorylated at Ser473), horseradish peroxidase (HRP)-linked anti-rabbit antibody, PI3K inhibitor LY294002, MEK1/2 inhibitor U0126, and cell lysis buffer were obtained from Cell Signaling Technology (Beverly, MA). All blue molecular weight marker was from Bio-Rad Laboratories (Hercules, CA).

**Experimental animals and conditions.** Juvenile rainbow trout of both sexes were obtained from Dakota Trout Ranch near Carrington, ND, and transported to North Dakota State University, where they were maintained in 800-liter tanks with recirculated (100% replacement volume per day) dechlorinated municipal water. The water was kept at 14°C with a 12:12-h photoperiod. Fish were fed to satiety twice per day with AquaMax Grower (PMI Nutrition International, Brentwood, MO), except 24 h before experiments. Animals were acclimated to laboratory conditions for at least 4 wk prior to experimentation. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, DC) and were approved by the North Dakota State University Institutional Animal Care and Use Committee.

At the time of experiments, fish were anesthetized in 0.05% (vol/vol) 2-phenoxyethanol, killed by transaction of the spinal cord, and hepatocytes were isolated by the in situ perfusion method described by Mommsen et al. (11). Isolated hepatocytes were allowed to recover in incubation medium [in mM: 136.9 NaCl, 5.4 KCl, 0.81 MgSO_4_, 0.44 KH_2PO_4_, 0.33 Na_2HPO_4_, 10 HEPES, 5 NaHCO_3_, 1.5 CaCl_2_, pH 7.6] with 2% (vol/vol) 2-phenoxyethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7 for 30 min at 50°C with occasional agitation) and washed twice with TBST, and the immunodetection protocol was repeated with the respective control antibody. It should be noted that doublet bands for ERK 1/2 and Akt were sometimes observed in Western blots and that such doublets were combined in quantitative analyses.

**HGH and IGF-I mRNA analysis.** Treated hepatocytes were homogenized and total RNA was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH) as specified by the manufacturer. Isolated RNA was dissolved in 25–75 μl of RNase-free deionized water, routinely quantified by UV (A260) spectrophotometry, then diluted with RNase-free, deionized water to 100 ng/μl. RNA samples were stored at −80°C until further evaluation; RNA quality was examined with the Agilent 2100 Bioanalyzer (Santa Clara, CA) prior to use. From 100 ng of total RNA, endogenous poly(A)^- RNA was reverse transcribed in a 5-μl reaction by using a TaqMan Reverse Transcription Reagent Kit containing MultiScribe Reverse Transcriptase and oligo(dT)_12-18 as a primer according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Reactions without reverse transcriptase were included as negative controls to exclude the possibility of contamination with genomic DNA. No amplification was detected in negative controls.

Steady-state mRNA levels of the two forms of GHR and of IGF-I were determined by real-time RT-PCR using TaqMan chemistry and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) as described previously (18, 26). Briefly, real-time PCR reactions were carried out for samples, standards, and no-template controls in a 10-μl volume containing 2 μl cDNA, 5 μl of TaqMan Universal PCR Master Mix, and 1 μl of each forward primer (HGH 1: 5’-TGAACCTTTCGATCATGGGACTAG-3’ [900 nM]), reverse primer (HGH 1: 5’-CGGGTACCCATATTGCATGCC-3’ [900 nM]), and probe (HGH 1: 5’-FAM-CAGAGGAGAGTGGGGAGGCAAC-3’ [100 nM]), and probe (IGF-I 1: 5’-CTACCCAGGTTGGTTGATGAA-3’ [900 nM]), and probe (IGF-I 2: 5’-CTACCCCTGTTTGATGGA-3’ [100 nM]).
for the mRNA species to be measured. Real-time PCR reactions were not multiplexed. Cycling parameters were as follows: 95°C for 10 min, and 45 cycles of 92°C for 15 s plus 60°C for 1 min. Sample copy number was calculated from the threshold cycle number (C_T) and relation of C_T to a gene-specific standard curve, followed by normalization to β-actin. No difference (P > 0.05) was observed in β-actin among the different treatment groups.

Statistics. Quantitative data are expressed as means ± SE. Statistical differences were estimated by ANOVA followed by Duncan’s multiple-range test. A probability level of 0.05 was used to indicate significance.

RESULTS

SS activates both the ERK and PI3 kinase-signaling pathways. Activation of cell signaling pathways was initially studied in SS-treated hepatocytes by examining tyrosine phosphorylation of endogenous proteins. Lysates from hepatocytes treated for various times or with different concentrations of SS-14-I were probed with a total phosphotyrosine antibody. As shown in Fig. 1, SS-14-I resulted in the phosphorylation of several proteins. The 42/44- and 60-kDa bands were tentatively identified as ERK 1/2 and Akt, respectively, on the basis of molecular weight; this identification was confirmed by immunoblotting analysis with specific antibodies.

The effects of SSs on activation of the ERK and PI3K pathways were studied on lysates probed with phospho-specific and control antibodies. SS-14-I treatment of hepatocytes induced ERK 1/2 phosphorylation in a time-dependent manner. Maximal phosphorylation of ERK 1/2 occurred within 5 min; pathway activation decreased thereafter but was sustained above control levels through 180 min (Fig. 2A). SS-14-I also stimulated ERK 1/2 phosphorylation in a concentration-dependent manner. Significant activation was observed at 1 ng/ml; the ED_{50} was ~40 ng/ml (Fig. 2B). SS-14-I also stimulated the phosphorylation of Akt. Significant Akt activation was not observed until 30 min after SS-14-I treatment but persisted for 180 min (Fig. 3A). Hepatocytes treated for 30 min with SS-14-I displayed significant Akt at 10 ng/ml; the ED_{50} was ~100 ng/ml (Fig. 3B).

The effectiveness of various isoforms of SS on the phosphorylation of ERK and Akt also was determined. ERK stimulation was affected differentially in hepatocytes treated with various SS isoforms. Isoforms derived from PPSS I, containing SS-14 at their COOH termini, all significantly stimulated the phosphorylation of ERK 1/2 (Fig. 4A). However, PPSS II-derived peptides, which have the [Tyr^{7},Gly^{10}] substitution at their COOH terminus, did not activate the ERK pathway. Whereas Akt activation also was affected differentially by SS isoforms, the pattern of activation...
was somewhat different compared with that observed for ERK activation. All isoforms derived from PPSS I (containing SS-14 at their COOH termini) stimulated Akt phosphorylation, especially the NH2-terminally extended forms (e.g., SS-26-I, SS-28-I) (Fig. 4B). In addition, PPSS II-derived isoforms (containing [Tyr7,Gly10]-SS-14 at their COOH termini) with the exception of shortest form, SS-14-II, stimulated phosphorylation of Akt (Fig. 4B).

Linkage of the ERK and PI3 kinase-signaling pathways to SS-mediated inhibition of GHR and IGF-I expression. Specific inhibitors of MEK and PI3K were used to examine the roles of the ERK and PI3K/Akt pathways on SS-inhibited expression of GHR and IGF-I mRNAs. The SS-14-I-induced activation of the ERK pathway was almost completely abolished by the MEK inhibitor U0126 (Fig. 5A). Similarly, SS-14-I-induced phosphorylation of Akt was prevented in hepatocytes treated with the Akt inhibitor, LY294002 (Fig. 5B). Together, these findings indicated that the conventional inhibitors of the ERK and PI3K/Akt pathways were effective in rainbow trout hepatocytes.

Consistent with previous findings, SS-14-I inhibited the steady-state levels of the two forms of GHR expressed in trout hepatocytes, GHR 1 and GHR 2. The MEK inhibitor U0126 blocked SS-14-I-inhibited expression of GHR 1 and GHR 2 mRNAs (Fig. 6). By contrast, the Akt inhibitor LY294002 was not able to rescue expression of GHRs in hepatocytes treated with SS-14-I (Fig. 7).
Also consistent with previous observations, SS-14-I was found to inhibit GH-stimulated IGF-I mRNA expression in isolated hepatocytes. Pretreatment of hepatocytes with the MEK inhibitor U0126 had no effect on SS inhibition of GH-stimulated IGF-I mRNA expression (Fig. 8A). By contrast, pretreatment of hepatocytes with the Akt inhibitor LY294002 partially overcame SS-14-I inhibition of GH-stimulated IGF-I expression (Fig. 8B).

DISCUSSION

Somatostatins previously were shown to inhibit GHR expression as well as basal and GH-stimulated IGF-I expression in the liver of rainbow trout (26, 27). The present results confirm the effects of SSs on GHR and IGF expression and support our starting hypothesis that SS exerts its GHR- and IGF-inhibiting actions by specific activation of ERK and Akt signaling pathways in trout hepatocytes. These findings extend our knowledge of actions of SS on the GH-IGF-I system and indicate that SS regulates organismal growth in both a central (pituitary) and peripheral (extrapituitary) manner.

The role of the ERK pathway on the hepatic expression of GHRs is supported by several observations. SS-14-I directly induced phosphorylation of ERK 1/2 in a time- and concentration-dependent manner. Blockade of the ERK pathway by U0126, a specific MEK inhibitor, prevented, at least in part, SS-14-I-induced phosphorylation of ERK. Blockade of the ERK pathway by U0126 rescued SS-inhibited expression of GHR 1 and GHR 2 mRNAs. Blockade of the PI3K pathway with LY294002, however, did not rescue SS-14-I-inhibited GHR expression. Together, these findings indicated that SS-inhibited GHR expression is mediated by ERK but not by PI3K/Akt.
The role of the PI3K/Akt pathway on hepatic expression of IGF-I is supported by several observations. SS-14-I directly induced phosphorylation of Akt in a time- and concentration-dependent manner. Blockade of the Akt pathway by LY294002 prevented, at least in part, SS-14-I-induced phosphorylation of Akt. Blockade of the PI3K pathway by LY294002 partially rescued the inhibition of GH-stimulated IGF-I mRNA expression by SS-14-I. Blockade of the ERK pathway with the MEK inhibitor U0126, however, did not block the inhibition of GH-stimulated IGF-I expression by SS-14-I. Together, these findings indicated that inhibition of GH-stimulated IGF-I expression by SS is mediated by the PI3K/Akt pathway but not by the ERK pathway.

The involvement of ERK and PI3K in SS-regulated GHR and IGF-I expression is consistent with previous findings. SS-14-I directly induced phosphorylation of Akt in a time- and concentration-dependent manner. Blockade of the Akt pathway by LY294002 prevented, at least in part, SS-14-I-induced phosphorylation of Akt. Blockade of the PI3K pathway by LY294002 partially rescued the inhibition of GH-stimulated IGF-I mRNA expression by SS-14-I. Blockade of the ERK pathway with the MEK inhibitor U0126, however, did not block the inhibition of GH-stimulated IGF-I expression by SS-14-I. Together, these findings indicated that inhibition of GH-stimulated IGF-I expression by SS is mediated by the PI3K/Akt pathway but not by the ERK pathway.

The present results also reveal structure-function relationships regarding SS-regulated GHR and IGF-I expression. For example, of the various forms of SS tested, only those derived from PPSS I (containing SS-14-I at its COOH terminus) activated ERK. By contrast, PPSS I-derived peptides as well as NH2-terminally extended PPSS II-derived peptides activated the PI3K/Akt pathway. The bases for these observed differ-
ences could be severalfold. First, fish, similar to mammals, possess multiple SSTRs (5, 10), and the liver of rainbow trout expresses a heterogeneous complement of SSTR subtypes. Second, the SSTR subtypes display differential ligand binding characteristics (10, 20). In rainbow trout, for example, SSTR 1A and SSTR 1B preferentially bind PPSS I-derived peptides over PPSS II-derived peptides (7). In addition, goldfish SSTR5A preferentially binds goldfish brain SS-28-II over PPI, whereas SSTR 2 stimulates a protein tyrosine phosphatase and phospholipase C (1, 2). Thus the observed differences in the relative efficacy of SS isoforms to activate the ERK and PI3K/Akt signaling pathways may be explained by numerous ligand-receptor-effector system interactions and/or ligand-dependent trafficking (15, 21). Further research on SSTR-effector system linkage is warranted to determine whether a particular SSTR subtype is responsible for ERK or Akt activation. It should be noted, however, that a broad range of both PPSS I-derived peptides and PPSS II-derived peptides, such as SS-25-II and SS-28-II, previously were found to inhibit GHR and GH-stimulated IGF-I expression in isolated trout hepatocytes (26, 27), suggesting that ERK and Akt do not exclusively mediate the actions of Ss on these processes.

Perspectives and significance. The present findings extend our knowledge of the extrapituitary actions of SS on the GH-IGF-I system and on the regulation of organisinal growth. Up to recently, SS was thought to regulate organisinal growth primarily at the level of pituitary by inhibiting the release of GH (4). Recent findings, however, revealed a number of important extrapituitary actions of SS on the GH-IGF-I system. At the level of the liver, SS reduced the sensitivity to GH by internalizing surface GHRs and by decreasing the synthesis of GHRs (26). In addition, SS inhibited basal and GH-stimulated IGF-I production (12, 27). At the level of the other target organs such as the gill, SS inhibited mRNA and functional expression of IGF-I receptors (25). Such local controls help explain differential responsiveness of tissues during embryonic and postembryonic growth. Local control also may be important for coordinating the growth-promoting actions of GH/IGF-I with other actions of GH/IGF-I (e.g., metabolic, reproductive, osmoregulatory) (cf. Refs. 5 and 23). For example, SS-induced attenuation of the growth-promoting actions of GH would be particularly adaptive and support the lipid catabolic actions of GH observed during food deprivation (14).

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REFERENCES

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