Regulation of Neuronal Nitric-oxide Synthase Activity by Somatostatin Analogs following SST5 Somatostatin Receptor Activation*

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Somatostatin receptor SST5 is an inhibitory G protein-coupled receptor that exerts a strong cytostatic effect on various cell types. We reported previously that the SST5 anti-proliferative effect results from the inhibition of mitogen-induced increases in intracellular cGMP levels and MAPK activity. This study was conducted to define the early molecular events accountable for the SST5-mediated anti-proliferative effect. Here, we demonstrate that, in Chinese hamster ovary cells expressing SST5 (CHO/SST5 cells), somatostatin inhibited cell proliferation induced by nitric oxide donors and overexpression of the neuronal nitric-oxide synthase (nNOS) protein isoform. Accordingly, nNOS activity and dimerization were strongly inhibited following SST5 activation by the somatostatin analog RC-160. In CHO/SST5 cells, nNOS was dynamically recruited by the SST5 receptor and phosphorylated at tyrosyl residues following RC-160 treatment. RC-160 induced SST5-p60°°° kinase complex formation and subsequent p60°°° kinase activation. Co-expression of an inactive p60°°° kinase mutant with SST5 blocked RC-160-induced nNOS phosphorylation and inactivation and prevented the SST5-mediated anti-proliferative effect. In CHO/SST5 cells, p60°°° kinase associated with nNOS to induce its inactivation by phosphorylation at tyrosyl residues following RC-160 treatment. Using recombinant proteins, we demonstrated that such phosphorylation prevented nNOS homodimerization. Next, surface plasmon resonance and mutation analysis revealed that p60°°° directly associated with nNOS phosphorylated Tyr°°°°. SST5-mediated inhibition of nNOS activity was demonstrated to be essential for the RC-160 anti-proliferative effect on pancreatic endocrine tumor-derived cells. We therefore identified nNOS as a new p60°°° kinase substrate essential for SST5-mediated anti-proliferative action.

Somatostatin is a neuropeptide that inhibits the secretion of many hormones, that acts as a neurotransmitter, and that negatively regulates cell proliferation (1). Somatostatin exerts its widespread physiological actions by stimulating specific membrane-associated receptors. Five distinct receptor subtypes (variably expressed in normal and neoplastic cells) have been characterized and cloned from human, mouse, and rat (2–5). An effect of somatostatin that has attracted attention in recent years is its potential role in inhibiting proliferating tissues. We have reported that stimulation of human SST2 and SST5 receptors leads to the inhibition of cell proliferation (6). SST2 recruits the protein-tyrosine phosphatase SHP-1 to the plasma membrane, and its subsequent activation correlates with a reduction of serum- and insulin-induced Chinese hamster ovary (CHO)2 DG44 cell proliferation (7, 8). Participation of p60°°° protein-tyrosine kinase, SHP-2 protein-tyrosine phosphatase, Ras-, Rap1-, and B-Raf-dependent ERK2 (extracellular signal-regulated kinase-2) activation in such an anti-proliferative effect was later demonstrated (9, 10). SST5 activation results in inhibition of soluble guanylate cyclase, decreased intracellular cGMP levels, MAPK activity, and cell proliferation of CHO-K1 cells stimulated by cholecystokinin (11). In mice, SST5 mediates somatostatin inhibition of pancreatic insulin secretion and contributes to the regulation of glucose homeostasis and insulin sensitivity (12). In addition, deficiency of SST5 leads to subtype-selective sexually dimorphic changes in the expression of both brain and pancreatic somatostatins (13).

Nitric oxide (NO) regulates a number of physiological processes, including smooth muscle contractility, platelet reactivity, neurotransmission, and the cytotoxic activity of immune cells. Three isoforms of nitric-oxide synthase (NOS) that generate NO from L-arginine have been characterized and named according to the cell type or conditions under which they were first identified. Both endothelial NOS and neuronal NOS (nNOS) are constitutively expressed, whereas expression of inducible NOS requires transcriptional activation. Because of its ubiquitous nature, inappropriate release of NO has been linked to a number of pathological conditions. In recent years, a growing body of evidence has depicted NO as a modulator of cell proliferation and/or survival of several normal and tumor systems such as vascular and airway smooth muscle cells, myo-

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§ The abbreviations used are: CHO, Chinese hamster ovary; MAPK, mitogen-activated protein kinase; NOS, nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; α-MEM, α-minimal essential medium; FCS, fetal calf serum; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; BH₄, tetrahydro-L-biopterin; DMEM, Dulbecco's modified Eagle's medium; SH2, Src homology 2; PBS, phosphate-buffered saline; GST, glutathione S-transferase; GPCR, G protein-coupled receptor; AT, angiotensin.
Regulation of nNOS Activity by Somatostatin Analogs

MATERIALS AND METHODS

Reagents—RC-160, a somatostatin analog, was a kind gift from Drs. A. V. Schally (Tulane University, New Orleans, LA). α-Minimal essential medium (α-MEM), Fungizone, streptomycin, penicillin, trypsin, and fetal calf serum (FCS) were purchased from Invitrogen. [γ-33P]ATP (3000 Ci/mmol) was purchased from Isotopichim (Garabogie, France). AG-50W-X8 resin (sodium form) was from Bio-Rad. [3H]-Arginine and the N-[14C]-2-mercaptoethanol were from VWR International SA, Berlin, Germany. L-citrulline, L-arginine, protein A-Sepharose beads, NaF, and leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Genetin (G418) was from Sigma. FuGENE 6 was from Roche. FuGENE 6 (3 μl/μl of DNA) was a generous gift from Dr. S. Roche (Centre de Recherches de Biochimie Macromoléculaire, CNRS, Montpellier, France). The primers used for site-directed mutagenesis of nNOS p60αcSH2 domain-binding sites (Y604F) were as follows: 5′-aact ctt cga ttc atc ctc gag gaa gta gc-3′ and 5′-gct act tcc tgc agg atg ttg aat cga gat tt-3′. Mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Roche Applied Science) following the manufacturer’s instructions.

For transient transfection, CHO/SST5 cells were grown for 8 h in α-MEM containing 5% FCS. After the α-MEM-containing serum was removed, cells were transfected in low serum-containing medium for 16 h with the pCMV5/nNOS and/or pSGT/Δp60αc vector at the concentrations indicated using FuGENE 6 (3 μl/μg of DNA) as described previously (41). Cells transfected with the pCMV5 vector were used as a control. Under these conditions of transient transfection, the percentage of cells transfected at 24 h was 48 ± 4 (mean ± S.E., n = 3) as quantified by green fluorescent protein expression (driven by the cytomegalovirus promoter) using a VisioLab 2000 image analysis system (Biocom, Paris, France) (41).

nNOS Activity—CHO/SST5 cells were plated in 60-mm diameter dishes at 50 × 10^3 cells/ml in α-MEM containing 5% FCS (5 ml/dish) before transient transfection, serum starvation, and treatment with RC-160 with or without other agents used at the times and concentrations indicated. nNOS activity was measured as described previously (39, 41). Briefly, cells were homogenized using a Dounce homogenizer (60 strokes at 4 °C) in 50 mM Tris buffer (pH 7.4) supplemented with 1 mM EDTA, 10% glycerol, 20 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.01% soybean trypsin inhibitor, and 0.1% CHAPS. 25 μg of cellular proteins were incubated for 15 min at 37 °C in 50 mM
Regulation of nNOS Activity by Somatostatin Analogs

Tris buffer (pH 7.4) containing 50 μM L-[14C]arginine (150,000 cpm, specific activity of 58.7 Ci/mmol), 10 mM β-NADPH, 1 mM dithiothreitol, 4 μM FMN, 4 μM FAD, 10 μM BH₄, 2 μg of calmodulin, and 1 mM CaCl₂ in a final volume of 200 μl. The reaction was terminated by addition of 500 μl of Dowex AG-50W-X8 (sodium form) pre-equilibrated in 50 mM (v/v) Hepes (pH 5.5) overnight at 4 °C. The mixture was gently agitated for 15 min at room temperature and centrifuged at 5000 × g for 5 min at 4 °C. Radioactivity in the supernatant was measured by liquid scintillation counting.

BON cells were plated for 24 h in 8-chamber slides at 50 × 10³ cells/chamber in DMEM containing 10% FCS (0.25 ml/chamber). Following overnight serum starvation, cells were washed twice with serum-free medium before treatment for 30 min with 10 nM RC-160. Real-time cell-associated NOS activity was monitored using an NOS fluorometric detection system (Sigma) with a Zeiss LSM510 confocal microscope at the times indicated following the manufacturer’s instructions.

Cell Growth Assay—CHO/SST5 cells were cultured in α-MEM containing 5% FCS and plated in 35-mm diameter dishes at 50 × 10³ cells/ml (2 ml/dish). After an overnight attachment phase and/or transient transfection, the cell medium was changed to serum-free α-MEM with or without different agents tested at the times and concentrations indicated. Cell growth was measured after 24 h of culture by cell counting using a Model ZM Coulter counter as described previously (6, 11). For cell growth assays, treatment with exogenous L-arginine was carried out in L-arginine-free α-MEM. For viability assays, BON cells were plated for 24 h in flat-bottomed 96-well plates at 10 × 10³ cells/well in DMEM containing 10% FCS (0.1 ml/well). Following overnight serum starvation, cells were treated in triplicate for 24 h with 100 μl of 10% FCS-containing medium in the presence or absence of RC-160 (10 nM) or the nNOS inhibitor α-guanidinoglutaric acid (0.1 mM). Cell viability was measured at 490 nm using the CellTiter 96 AQueous One Solution cell proliferation assay (Promega Corp.) following the manufacturer’s instructions.

p60src Kinase Activity Assay—CHO/SST5 cells were plated in 100-mm diameter dishes at 75 × 10³ cells/ml (10 ml/dish) in α-MEM containing 5% FCS. After an 18-h period of serum deprivation and/or transient transfection, cells were not treated with RC-160 at the times and concentrations indicated. CHO/SST5 cells were then washed twice with 20 mM Tris buffer (pH 7.4) containing 150 mM NaCl and 0.5 mM orthovanadate (buffer A). p60src activity was quantitated as described previously (45) with some minor modifications. Briefly, cells were scrapped and solubilized at 4 °C for 15 min in buffer A containing 1% Nonidet P-40, 10 mM NaF, 2.5 mM EDTA, 20 μg leupeptin, and 1% aprotinin. Supernatant-containing soluble proteins were obtained by centrifugation at 13,000 × g for 10 min at 4 °C. Soluble proteins (250 μg) were incubated for 1.5 h at 4 °C without agitation with 0.5 μg of anti-p60src kinase antibody or preimmune serum prebound to protein A-Sepharose beads prewashed with buffer A as a control. The beads were then washed once with buffer A and once with 20 mM Hepes (pH 7.4) containing 10 mM MnCl₂ and 1.5 mM orthovanadate (buffer B). The reaction was initiated by addition of 20 μl of buffer B containing 50 μM ATP, 5 μCi of [γ-32P] ATP, and 4 μg of heat-denatured enolase. The reaction was carried out for 15 min at 30 °C and terminated by addition of 30 μl of protein loading buffer containing 3% SDS and β-mercaptoethanol. Samples were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were air-dried and exposed on a PhosphorImager screen for 16 h. Phosphorylated enolase was visualized using a PhosphorImager (GE Healthcare) and quantified by image analysis using ImageQuant software (GE Healthcare).

Immunoprecipitation and Immunoblotting—CHO/SST5 cells were plated in 100-mm diameter dishes at 100 × 10³ cells/ml (10 ml/dish) for 8 h in α-MEM containing 5% FCS before transient transfection and serum starvation. BON cells were plated in 100-mm diameter dishes at 150 × 10³ cells/ml (10 ml/dish) for 16 h in DMEM containing 10% FCS. Cells were or were not treated with RC-160 with or without other agents tested at the times and concentrations indicated. Cells were then washed twice with phosphate-buffered saline (PBS) and scraped on ice with 50 mM Tris buffer (pH 7.4) containing 140 mM NaCl, 2 mM EDTA, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride (buffer C) in the presence of 1.5% CHAPS, 0.05% soybean trypsin inhibitor, and 0.5 mM sodium orthovanadate. The mixture was gently agitated for 30 min at 4 °C and thereafter centrifuged at 13,000 × g for 20 min at 4 °C. Soluble proteins (500 μg to 1 mg) were incubated overnight at 4 °C with a 1:100 dilution of polyclonal anti-nNOS, monoclonal anti-phosphotyrosine, polyclonal anti-p60src kinase, or polyclonal anti-SST5 antibody prebound to protein A-Sepharose beads prewashed with buffer C in the presence of 0.1% CHAPS, 0.01% soybean trypsin inhibitor, and 0.3% bovine serum albumin (buffer D). Immunoprecipitation with preimmune serum was used as a control. Following incubation, beads were washed twice with buffer D and resuspended in 35 μl of protein loading buffer containing 3% SDS prior to electrophoresis. For immunoblotting, solubilized (50 μg) or immunoprecipitated proteins were resolved on 7.5 or 10% SDS-polyacrylamide gels before transfer to nitrocellulose membranes. After blocking at room temperature for 2 h in PBS (pH 7.4), 0.05% Tween 20, and 5% dry milk, blots were immunoprobed overnight at 4 °C with monoclonal anti-nNOS (1:1000), polyclonal anti-nNOS (1:500), monoclonal anti-phosphotyrosine (1:1000), polyclonal anti-human SST5 (1:500), or anti-p60src kinase (1:500) antibody diluted in PBS (pH 7.4), 0.05% Tween 20, and 1% dry milk. The membrane was incubated in 5% nonfat dry milk, incubated with a 1:500 dilution of anti-human SST5 (1:500) antibody, or anti-p60src kinase antibody and by image analysis using ImageQuant software. To visualize nNOS dimerization, low temperature SDS-PAGE was performed as described (41). Briefly, cells were solubilized for 30 min at 4 °C in 50 mM Tris buffer (pH 7.4) containing 140 mM NaCl, 5 mM MgCl₂, 0.5 mM orthovanadate, 0.05% soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 1% CHAPS and then centrifuged at
13,000 \times g for 20 min at 4 °C. After addition of protein loading buffer containing 3% SDS under nonreducing conditions, 100 µg of solubilized proteins were resolved by discontinuous gradient 5–15% SDS-PAGE performed at a constant current of 30 mA. Gels and buffers were equilibrated at 4 °C prior to electrophoresis, which was performed below 15 °C. Immunoblotting was performed using monoclonal antihuman nNOS antibody as described previously (41).

**Surface Plasmon Resonance**

The binding kinetics of glutathione S-transferase (GST) fusion proteins were measured with a Biacore 3000 biosensor. Binding of GST fusion protein mass to immobilized peptide was quantified as resonance units, where 1000 resonance units represent 1 ng of protein bound per mm² of flow cell surface. Samples were diluted in Hank’s balanced salt running buffer. 50 resonance units of biotinylated nNOS peptides phosphorylated at either tyrosyl residue 604 or 1336 were immobilized on streptavidin-coated carboxymethylated dextran chips and then subjected to 1–100 nM GST-p60 src SH2 domain fusion protein (a kind gift of Dr. S. Roche) at a flow rate of 20 µl/min at 25 °C. GST fusion protein binding to the non-phosphorylated biotinylated peptide was subtracted. Binding constants were calculated from the titration curves using the global fitting routine software BIAevaluation Version 4.0.1.

**In Vitro Assays for Recombinant nNOS Dimerization and Phosphorylation by Recombinant p60 src**

Low temperature-PAGE using recombinant nNOS was conducted as described previously (46) with some modifications. Briefly, 500 ng of recombinant rat nNOS (purity >95%, specific activity of 858 units/mg of protein; Sigma) were incubated in 50 mM Hepes (pH 7.4) containing 1 mM BH₄ for 10 min at 37 °C in a final volume of 10 µl. To induce nNOS homodimerization, BH₄ was raised to 100 mM. Samples were subsequently incubated with 2 units of human purified active p60 src (Calbiochem) diluted in 50 mM Hepes (pH 7.5) containing 0.1 mM EDTA, 0.015% Tween 20, 0.1 mg/ml bovine serum albumin, and 0.2% β-mercaptoethanol as described previously (47). p60 src activity was initiated by addition of 10 µl of ATP mixture (0.15 mM ATP and 30 mM MgCl₂) diluted in 50 mM Hepes (pH 7.5) containing 0.1 mM EDTA and 0.015% Tween 20 and further incubation at 30 °C for 30 min. Incubations were terminated by addition of 40
Regulation of nNOS Activity by Somatostatin Analogs

µl of Laemmli buffer containing 0.125 M Tris-Cl (pH 6.8), 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (w/v) glycerol, and 0.02% (w/v) bromphenol blue, followed by storage on ice. As a monomerization control, samples were heat-denatured at 95 °C for 5 min. To visualize nNOS dimerization, samples were subjected to low temperature SDS-PAGE, followed by Western blotting for nNOS as described. To visualize nNOS phosphorylation, samples were first subjected to immunoprecipitation using anti-phosphotyrosine antibody before low temperature SDS-PAGE, followed by Western blotting for nNOS as described.

RESULTS

Cell Proliferation Induced by Nitric Oxide Is Inhibited following SST5 Somatostatin Receptor Activation—To obtain direct evidence for nNOS implication in the SST5-mediated anti-proliferative effect, we first transiently transfected cells with recombinant nNOS cDNA. Successful expression of nNOS and subsequent production of NO in CHO/SST5 cells were verified by Western blotting and by NOS activity, respectively (Fig. 1, A and B). Under these conditions, transgene expression was routinely observed in 48 ± 4% (mean ± S.E., n = 3) of the cell population as quantified by green fluorescent protein expression (41) (data not shown). As shown in Fig. 1C, we found that cell proliferation was stimulated after transient transfection with nNOS DNA (+41.5 ± 2.5%) compared with control cells. A similar cell proliferation increase was observed when cells were treated with endogenous or exogenous sources of NO (0.1 µM sodium nitroprusside (+62 ± 4%) and 100 µM L-arginine (+55 ± 5%)) compared with control cells.

Next, we investigated whether activated SST5 inhibits nNOS/NO-induced cell proliferation. CHO/SST5 cells were treated for 24 h with NO donors in the presence of the somatostatin analog RC-160. As shown in Fig. 1C, treatment of CHO/SST5 cells with 10 nM RC-160 strongly inhibited nNOS/NO-induced cell proliferation. Taken together, these results demonstrated that 1) cell proliferation of CHO/SST5 cells was induced by endogenous and exogenous NO donors and that 2) treatment of CHO/SST5 cells with RC-160 strongly inhibited nNOS/NO-induced cell proliferation.

nNOS Activity Is Inhibited following SST5 Somatostatin Receptor Activation—We then investigated whether activated SST5 inhibits nNOS activity in CHO/SST5 cells. CHO/SST5 cells were transiently transfected with nNOS DNA before treatment with 10 nM RC-160. As shown in Fig. 2A, SST5 somatostatin receptor activation resulted in a time-dependent inhibition of nNOS-induced NO production, with a maximal effect observed after 30 s of treatment with RC-160 (−74 ± 15%). Because the nNOS enzyme is known to be active as a dimer (48), nNOS homodimerization status was monitored in CHO/SST5 cells by low temperature SDS-PAGE. As shown in Fig. 2B, the proportion of the inactive monomeric form of transfected nNOS was increased by 82 ± 6% 30 s following addition of 10 nM RC-160 and was stable up to 15 min of treatment. Taken together, these results suggest that RC-160 inhibits nNOS activity and prevents dimerization of the enzyme.

SST5 Associates with nNOS to Induce Its Phosphorylation at Tyrosyl Residues—A recent study indicates that a variety of proteins directly interact with nNOS and regulate its activity (49). We examined whether SST5 interacts with nNOS in CHO/SST5 cells transiently expressing nNOS by immunoblotting anti-nNOS immunoprecipitates with anti-SST5 antibody. SST5 was detected in resting cells within anti-nNOS immunoprecipitates (Fig. 3, A and B); and inversely, nNOS was detected in resting cells within anti-SST5 immunoprecipitates (Fig. 3, C
Preimmune antiserum was ineffective in immunoprecipitating protein complexes. Exposure of CHO/SST5 cells to RC-160 treatment increased the amount of the SST5 receptor immunoprecipitated with nNOS in a time-dependent manner to a maximal 15–30 s following RC-160 treatment (4.3-fold and 2.5-fold over basal levels in anti-nNOS and anti-SST5 immunoprecipitates, respectively) (Fig. 3, B and D). Of note, interaction of the SST5 receptor with endogenous nNOS was not detectable in anti-SST5 immunoprecipitates (data not shown).

We demonstrated previously that nNOS activity is inversely correlated to its state of phosphorylation at tyrosyl residues; upon recruitment, members of the protein-tyrosine phosphatase family such as SHP-1 and SHP-2 dephosphorylate and activate nNOS (39, 41). We therefore explored whether RC-160 regulates the level of nNOS tyrosine phosphorylation following SST5 activation. As
Regulation of nNOS Activity by Somatostatin Analogs

A

**FIGURE 4.** p60src kinase is involved in RC-160-mediated inhibition of cell proliferation in CHO cells expressing SST5. 

A. CHO/SST5 cells were cultured until they were subconfluent and cotransfected with 1 μg of pCMV5/nNOS and pSGT/Δp60src plasmids or control plasmid. Cells were treated in the absence of serum with 10 nM RC-160 (shaded bars) for 24 h. Cell proliferation was assayed by cell counting (mean ± S.E. of three experiments performed in triplicate). **, p < 0.01.

B. CHO/SST5 cells were transiently transfected with pSGT/Δp60src DNA. 24 h later, cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting with anti-p60src antibody. Filters were reprobed with anti-SST5 antibody to ensure comparable amounts of protein. Results are representative of two immunoblottings, each performed after different sets of cell transfection.

shown in Fig. 3 (A and B), nNOS was phosphorylated at tyrosyl residues in resting cells. Treating CHO/SST5 cells with 10 nM RC-160 strongly increased nNOS tyrosine phosphorylation in a time-dependent manner. This effect was maximal after 30 s of treatment (4.9 ± 0.25-fold over basal levels). The kinetics of nNOS phosphorylation were in accordance with RC-160-induced SST5-nNOS complex formation and RC-160 inhibitory effect on nNOS activity. Taken together, these data demonstrated that SST5 associates with nNOS in resting cells. RC-160 treatment promoted a rapid and transient increase in SST5-nNOS complexes associated with the phosphorylation of nNOS at tyrosyl residues.

p60src Is Required for the SST5-mediated Anti-proliferative Effect—Increasing evidence suggests that G protein-coupled receptor (GPCR) signals engage protein-tyrosine kinases, including non-receptor tyrosine kinases such as Src family kinases (50–53). In an attempt to determine whether p60src catalyzes nNOS tyrosyl phosphorylation and plays a role in the SST5-mediated anti-proliferative effect, CHO/SST5 cells were transiently transfected using a catalytically inactive mutant of p60src in which Lys295 was replaced with Met (44). Successful expression of the p60src mutant in CHO/SST5 cells was verified by Western blotting (Fig. 4B). Transient transfection of CHO/SST5 cells with the p60src kinase mutant did not significantly modify nNOS-induced CHO/SST5 cell proliferation (Fig. 4A). However, the p60src mutant strongly antagonized the RC-160 inhibitory effect on nNOS-transfected CHO/SST5 cell proliferation (Fig. 4A). p60src activity was quantified by Src kinase-specific substrate enolase phosphorylation. As shown in Fig. 5D, p60src activity was very low to undetectable at the basal level in CHO/SST5 cells. Treatment with RC-160 strongly induced p60src activity in a time-dependent fashion to a maximal 30 s following treatment (7.9 ± 0.2-fold over basal levels). This latter result was further confirmed by the increase of SST5 in anti-p60src immunoprecipitates from RC-160-treated CHO/SST5 cells (Fig. 5C).

To test whether the activated SST5 receptor regulates p60src activity, CHO/SST5 cells were treated with RC-160, and cell lysates were immunoprecipitated with anti-p60src antibody. p60src activity was quantified by Src kinase-specific substrate enolase phosphorylation. As shown in Fig. 5D, p60src activity was very low to undetectable at the basal level in CHO/SST5 cells. Treatment with RC-160 strongly induced p60src activity in a time-dependent fashion to a maximal 30 s following treatment (7.9 ± 0.2-fold over basal levels). The kinetics of p60src activity correlated well with RC-160-induced SST5-p60src complex formation. Taken together, these data demonstrated that SST5 associated with p60src in resting cells. RC-160 treatment promoted a rapid increase in SST5-p60src complexes and the subsequent activation of p60src.

nNOS Is a New p60src Kinase Substrate—We next examined whether p60src kinase interacts with nNOS in CHO/SST5 cells transiently transfected with nNOS by immunoblotting anti-SST5 receptor with anti-p60src antibody. 40 nM p60src activity was quantified by Src kinase-specific substrate enolase phosphorylation. As shown in Fig. 5A, p60src activity was very low to undetectable at the basal level in CHO/SST5 cells. Treatment with RC-160 increased the amount of p60src kinase immunoprecipitated with SST5 in a time-dependent fashion to a maximal 30 s following treatment (7.9 ± 0.2-fold over basal levels). This latter result was further confirmed by the increase of SST5 in anti-p60src immunoprecipitates from RC-160-treated CHO/SST5 cells (Fig. 5C).

SST5 Associates with and Activates p60src Kinase—We next examined whether p60src kinase interacts with the SST5 receptor by immunoblotting anti-SST5 immunoprecipitates with anti-p60src antibody. As shown in Fig. 5A, p60src kinase was detected within anti-SST5 immunoprecipitates in resting cells, whereas preimmune antiserum was ineffective in immunoprecipitating protein complexes. Exposure of CHO/SST5 cells to RC-160 increased the amount of p60src kinase immunoprecipitated with SST5 in a time-dependent fashion to a maximal 30 s following treatment (6.2 ± 0.2-fold over basal levels). This latter result was further confirmed by the increase of SST5 in anti-p60src immunoprecipitates from RC-160-treated CHO/SST5 cells (Fig. 5C).

To test whether the activated SST5 receptor regulates p60src activity, CHO/SST5 cells were treated with RC-160, and cell lysates were immunoprecipitated with anti-SST5 antibody. As shown in Fig. 5, D, p60src activity was very low to undetectable at the basal level in CHO/SST5 cells. Treatment with RC-160 strongly induced p60src activity in a time-dependent fashion to a maximal 30 s following treatment (7.9 ± 0.2-fold over basal levels). The kinetics of p60src activity correlated well with RC-160-induced SST5-p60src complex formation. Taken together, these data demonstrated that SST5 associated with p60src in resting cells. RC-160 treatment promoted a rapid increase in SST5-p60src complexes and the subsequent activation of p60src.

The kinetics of p60src association with nNOS correlated well with RC-160-induced SST5-p60src complex formation and dephosphorylation of nNOS at tyrosyl residues.
To obtain evidence that activated SST5 may inhibit nNOS in CHO/SST5 cells by stimulating p60src kinase, cells were transiently transfected with nNOS and the catalytically inactive p60src kinase mutant. Fig. 6C demonstrates that p60src was critical for SST5-induced inhibition of nNOS activity because expression of the p60src mutant completely reversed RC-160-mediated inhibition of nNOS activity. Furthermore, phosphorylation of nNOS induced by RC-160 treatment of CHO/SST5 cells was abolished after transfection of the inactive p60src kinase mutant (Fig. 6D). Interestingly, association of wild-type and mutant p60src proteins with nNOS upon RC-160 treatment of CHO/SST5 cells was similar (Fig. 6E). Next, we used recombinant proteins in vitro to obtain direct evidence of p60src-mediated phosphorylation and inhibition of nNOS activity. We first confirmed that in vitro nNOS homodimerization can be induced using 100 μM BH4, followed by incubation at 37 °C for 10 min (Fig. 6F, lane 3) as described previously (46). As expected, nNOS dimers were resistant to p60src kinase treatment (Fig. 6F, lane 4). Thus, nNOS was first incubated with recombinant p60src in the presence of ATP at 30 °C for 30 min prior to incubation at 37 °C for 10 min with or without 100 μM BH4. We found that preincubating nNOS monomers with p60src strongly antagonized BH4-induced nNOS homodimerization (Fig. 6F, lane 5). Immunoprecipitation and Western blotting demonstrated that recombinant p60src phosphorylated recombinant nNOS monomers at tyrosyl residues in vitro (Fig. 6G, lane 3). Again, p60src did not phosphorylate already established nNOS dimers (Fig. 6G, lanes 2 and 3). In the absence of p60src, nNOS was not phosphorylated at tyrosyl residues (Fig. 6G, lane 1). Taken together, these results showed that SST5 stimulated p60src activity to induce nNOS monomer phosphorylation at tyrosyl residues and subsequent inactivation. Together, these data describe nNOS as a novel p60src kinase substrate essential to SST5-mediated growth arrest.
FIGURE 6. p60src is essential to SST5-induced nNOS inactivation by phosphorylation and the subsequent anti-proliferative effect. A, CHO/SST5 cells were cultured until they were subconfluent and transfected with 1 μg of pCMV5/nNOS plasmid. 24 h later, cells were or were not incubated at 37 °C for the indicated times with 10 nM RC-160 in the absence of serum. nNOS was immunoprecipitated (i.p.) before sequential immunoblotting with anti-p60src kinase and anti-nNOS antibodies. PI, preimmune. B, immunoreactive proteins were quantified by image analysis using ImageQuant software. Results are representative of three immunoblottings, each performed after different sets of cell transfection and RC-160 treatments. *, p < 0.05; **, p < 0.01. C, CHO/SST5 cells were cultured until they were subconfluent and cotransfected with 1 μg of pCMV5/nNOS and control plasmids or with 1 μg of pCMV5/nNOS and pSGT/Δp60src plasmids. Cells transfected with 2 μg of pCMV5 vector were used as a control. 24 h later, cells were or were not treated with 10 nM RC-160 for 30 s, and NOS activity was measured. The basal levels of NO production were 8.2 ± 1.6 and 35.6 ± 3.7 pmol of citrulline/mg/min in CHO/SST5 cells and pCMV5/nNOS-transfected cells, respectively. Results are expressed as the percent NO production in nNOS-transfected cells alone and are the mean ± S.E. of three separate experiments performed in duplicate. **, p < 0.01. D and E, CHO/SST5 cells were cultured until they were subconfluent and cotransfected with 1 μg of pCMV5/nNOS and control plasmids or with 1 μg of pCMV5/nNOS and pSGT/Δp60src plasmids. Cells transfected with 2 μg of pCMV5 vector were used as a control. 24 h later, cells were or were not incubated for 30 s with 10 nM RC-160 in the absence of serum. nNOS (D) and p60src (E) were immunoprecipitated before immunoblotting with anti-nNOS and anti-p60src antibodies. Results are representative of two immunoblottings, each performed after different sets of cell transfection and RC-160 treatments. F, recombinant rat nNOS was incubated at 37 °C for 10 min in the presence (lane 3) or absence (lanes 1 and 2) of 100 μM BH4. 2 units of human purified active p60src were added, and incubation was continued for 30 min at 30 °C (lane 4). Alternatively, nNOS was first incubated with active p60src in low BH4-containing buffer at 30 °C for 30 min, followed by incubation at 37 °C for 10 min in the absence (lane 5) or presence (lane 6) of 100 μM BH4. Incubations were terminated by addition of 40 μl of Laemmli buffer, and samples were stored on ice (lanes 2–5) or heat-denatured as a control (lane 1). Samples were resolved by low temperature discontinuous gradient SDS-PAGE and analyzed by immunoblotting with anti-nNOS antibody. Results are representative of two immunoblottings, each performed after different in vitro reactions. G, immunoreactive proteins were quantified by image analysis using ImageQuant software. H, to visualize nNOS phosphorylation, samples corresponding to lanes 3, 4, and 6 in panel F (lane 1–3, respectively) were first subjected to immunoprecipitation using anti-phosphotyrosine (P-Tyr) antibody (4G10) before low temperature SDS-PAGE, followed by Western blotting for nNOS. Results are representative of two immunoblottings, each performed after different in vitro reactions.
**Regulation of nNOS Activity by Somatostatin Analog**s

*JOURNAL OF BIOLOGICAL CHEMISTRY*

**p60src Directly Associates with nNOS Phosphorylated Tyrosine 604—**Upon ligand binding, receptor tyrosine kinases undergo autoposphorylation of selected tyrosine residues, which become docking sites for adaptor proteins and enzymes that propagate the signal. Typically, these signaling proteins contain modular domains such as p60src SH2 or phosphotyrosine-binding domains that recognize only phosphotyrosines surrounded by specific sequences. The optimal consensus sequence for the SH2 domain of p60src has been determined to be pYEEI by screening of a phosphopeptide library (54). Sequence analysis revealed that nNOS contains two putative binding sites for p60src SH2 domains at amino acids 604 (CDNRSRpYNILEE, peptide 1) and 1336 (LQEQLAESVpYRALKE, peptide 2). Therefore, we investigated whether p60src directly interacts with nNOS. For this purpose, recombinant GST fusion proteins containing p60src SH2 domains were produced. We used surface plasmon resonance to measure real-time interaction between p60src SH2 domains and nNOS putative p60src-binding domains. Tyrosine-phosphorylated biotylated peptides 1 and 2 were immobilized on streptavidin sensor chips. As shown in Fig. 7A (left panel), p60src SH2 domains strongly bound to phosphorylated peptide 1, whereas no significant binding to phosphorylated peptide 2 could be detected (right panel). Of note, no binding was detected when non-phosphorylated peptides were immobilized on the sensor chips. Using increasing concentrations of recombinant GST fusion proteins containing p60src SH2 domains, we found that nNOS phosphorylated Tyr604 and p60src SH2 domains interacted with high affinity (K_D = 21 nm).

We next devised an nNOS mutant in which Tyr604 was replaced with phenylalanine (pCMV5/nNOS(Y604F)). CHO/SST5 cells were transiently transfected with wild-type nNOS or nNOS(Y604F). Successful expression of both proteins in CHO/SST5 cells was verified by Western blotting (Fig. 7B). We next measured the ability of the nNOS mutant to interact with p60src and SST5 by immunoprecipitation. Both wild-type and mutant nNOS were faintly detected within anti-p60src and anti-SST5 immunoprecipitates in resting cells (Fig. 7, C and D, respectively). As expected, exposure of CHO/SST5 cells to 10 nm RC-160 strongly increased the amount of nNOS and SST5 immunoprecipitated with wild-type p60src (Fig. 7, C and D, respectively). However, mutation of Tyr604 strongly inhibited RC-160-induced nNOS binding to p60src and SST5 (Fig. 7, C and D, respectively). Finally, RC-160 failed to inhibit mutant nNOS-induced elevation of intracellular NO (Fig. 7E). Taken together, these results demonstrated that Tyr604 mediated nNOS binding to p60src and that such an interaction was critical to SST5-mediated inhibition of nNOS activity.

The SST5-mediated Anti-proliferative Action Initiated by p60src-dependent nNOS Phosphorylation and Inactivation Is Effective in Pancreatic Tumor Cells—Because somatostatin and somatostatin analogs are well-characterized inhibitors of gastroenteropancreatic neuroendocrine tumor cell secretion and growth in vivo and in vitro (55–59), we investigated whether SST5-mediated inhibition of nNOS activity following p60src recruitment might account for inhibition of pancreatic neuroendocrine tumor-derived cell proliferation. BON cells, isolated from human pancreatic carcinoid (60), endogenously expressed the SST5 receptor, p60src, and nNOS, but not endothelial NOS, as ascertained by reverse transcription-PCR and Western blotting (data not shown). We examined whether SST5 interacts with p60src in BON cells by immunoblotting anti-SST5 immunoprecipitates with anti-p60src antibody. As shown in Fig. 8A, p60src was detected in resting cells within anti-SST5 immunoprecipitates, whereas preimmune antiserum was ineffective in immunoprecipitating protein complexes (data not shown). Exposure of BON cells to RC-160 treatment increased the amount of p60src immunoprecipitated with SST5 1 min following RC-160 treatment (Fig. 8A). Next, we examined whether SST5 interacts with nNOS in BON cells by immunoblotting anti-nNOS immunoprecipitates with anti-SST5 antibody. As shown in Fig. 8B, SST5 was detected in resting cells within anti-nNOS immunoprecipitates, whereas preimmune antiserum was ineffective in immunoprecipitating protein complexes (data not shown). Exposure of BON cells to RC-160 treatment increased the amount of the SST5 receptor immunoprecipitated with nNOS 1 min following RC-160 treatment (Fig. 8B). We therefore explored whether RC-160 could regulate the level of nNOS tyrosine phosphorylation following SST5 activation in BON cells. As shown in Fig. 8B, nNOS was weakly phosphorylated at tyrosyl residues in resting cells. Treating BON cells for 1 min with 10 nm RC-160 strongly increased nNOS tyrosine phosphorylation. Taken together, these data demonstrated that SST5 associated with nNOS and p60src in resting BON cells. RC-160 treatment promoted a rapid increase in SST5-nNOS and SST5-p60src complexes associated with the phosphorylation of nNOS at tyrosyl residues.

We next investigated whether activated SST5 inhibits nNOS activity in BON cells. BON cells were incubated for 30 min with 10 nm RC-160. Real-time NO production was measured in live BON cells. As shown in Fig. 8C, SST5 somatostatin receptor activation strongly and irreversibly antagonized nNOS-induced NO production in BON cells compared with control cells. Treating BON cells with either RC-160 (10 nm) or the nNOS inhibitor α-guanidinoglutaric acid (0.1 μM) drastically inhibited cell proliferation (Fig. 8D). Interestingly, no synergistic inhibitory effect on cell proliferation was measured when RC-160 and α-guanidinoglutaric acid were concomitantly administrated to BON cells. Taken together, these results demonstrated that the endogenous SST5 receptor might interact with p60src upon RC-160 stimulation of human gastroenteropancreatic neuroendocrine tumor cells to inhibit nNOS activity and subsequent cell proliferation.

**DISCUSSION**

We have reported the anti-proliferative action of the somatostatin analog RC-160 in CHO-K1 cells stably transfected with the human somatostatin receptor SST5 (6). These results were further extended by the demonstration of the SST5-mediated inhibitory effect of somatostatin on mitogen-induced increases in intracellular cGMP levels, MAPK activity, and subsequent cell proliferation (11). The present study was conducted to identify the early molecular events involved in the somatostatin anti-proliferative effect mediated by the SST5 receptor subtype.
Regulation of nNOS Activity by Somatostatin Analogs

A

GST-p60srcSH2

Peptide 1: CDNSRYNILEEV

Response (RU)

Time (msec)

Peptide 2: LQEQLAESVYRALKE

Response (RU)

Time (msec)

B

160 kDa -

105 kDa -

Blot: nNOS

Blot: sst5

pCMV5

pCMV5(nNOS)

pCMV5(nNOSY604F)

C

i.p.: p60src

Blot: nNOS

Blot: p60src

pCMV5(nNOS)

pCMV5(nNOSY604F)

RC-160 (10nM)

D

i.p.: sst5

160 kDa -

105 kDa -

Blot: nNOS

Blot: sst5

pCMV5

pCMV5(nNOS)

pCMV5(nNOSY604F)

RC-160 (10nM)

E

NOS activity

pmol citrulline·mg·min⁻¹

pCMV5

pCMV5(nNOS)

pCMV5(nNOSY604F)

RC-160 (10nM)
Regulation of nNOS Activity by Somatostatin Analogs

NO is a ubiquitous, gaseous, endogenous messenger molecule that participates in a wide variety of physiological and physiopathological processes partially through elevation of intracellular cGMP levels. Previous work from our laboratory demonstrated that NO originating from the endogenous nNOS isoform acts positively on CHO-K1 cell proliferation (41). Here, we extended these results by the demonstration of the proliferative effect of the NO donor sodium nitroprusside, the NOS substrate l-arginine, and nNOS protein overexpression on CHO/SST5 cells. Even if the NO anti-proliferative effect on multiple cell types is well documented (14–25), the present work represents a new addition to the growing list of studies describing NO as involved in stimulating cell proliferation (28–30, 38, 41).

Thus, the role of NO in the SST5-promoted growth inhibition signal was further investigated. Treating SST5-bearing CHO cells with the somatostatin analog RC-160 strongly reduced cell proliferation induced by NO donors, probably through the inhibition of cGMP-induced MAPK activity as we reported previously (11). Furthermore, RC-160 treatment drastically inhibited cell proliferation following nNOS expression. These data suggest that somatostatin acting through the SST5 receptor could inhibit NO production originating from nNOS. This possibility was confirmed by the study of nNOS activity in CHO/SST5 cells following treatment with RC-160. We observed that RC-160 induced a rapid inhibition of nNOS activity and strongly inhibited the formation of active nNOS dimers in favor of the accumulation of inactive nNOS monomers. We demonstrated that nNOS was constitutively associated with SST5 in CHO/SST5 cells and that SST5-nNOS complex formation was up-regulated after somatostatin binding to SST5.

GPCRs that have been shown to positively regulate nNOS activity include α-adrenoreceptors and glutamate, bradykinin, endothelin-1, purinergic, opioid, cannabinoid, serotonin, and cholecystokinin receptors (41, 61, 62). To our knowledge, the capacity of SST5 to bind and inhibit nNOS is the unique demonstration of negative coupling between a GPCR and the nNOS isoform. Interestingly, we reported the positive coupling of the SST2 somatostatin receptor and nNOS in the SST2-mediated anti-proliferative effect (39). The activated SST2 receptor stimulates the tyrosine phosphatase SHP-1, which results in nNOS tyrosyl dephosphorylation and activation. Conversely, somatostatin was found to be a powerful inhibitor of tumor angiogenesis through SST3-mediated inhibition of both endothelial NOS and MAPK (63). In addition, a recent study linked SST2 activation to inducible NOS-derived production inhibition (64). Such bimodal regulation of NO production by different receptor subtypes has been observed following angiotensin (AT) II treatment. Through its AT1 receptor, AT II stimulates the long-term increase in several membrane components of NADPH oxidase and indirectly inhibits NO production, leading to endothelium dysfunction. Conversely, the AT2 receptor counterbalances the deleterious effect of AT II-induced AT1 receptor stimulation through bradykinin and direct NOS stimulation (for review, see Ref. 65). Recently, receptor subtype-selective inhibition of long-term NO production by somatostatin was reported (40).

This study provides the first demonstration that somatostatin receptor subtypes can directly act antagonistically in regulating nNOS activity. Such opposite regulation of NO by somatostatin with widespread receptor distribution can achieve functional selectivity in different tissues and physiological states.

The activity of the constitutive NOS isoforms such as nNOS is critically controlled by calcium, which promotes binding of calmodulin to the enzymes and subsequent activation. We demonstrated previously that RC-160 inhibits cholecystokinin-induced mobilization of intracellular calcium in CHO/SST5 cells (6). However, this effect is not implicated either in cholecystokinin-induced nNOS activation or in the anti-proliferative effect mediated by the SST5 somatostatin receptor in these cells (6, 11). Variations in the level of phosphorylation have been recently suggested to alter conformation, activity, and/or protein coupling of nNOS. In this context, phosphorylation of nNOS at Ser<sup>647</sup> by calcium/calmodulin-dependent protein kinase II reduces nNOS activity, whereas dephosphorylation of the same residue by protein phosphatase-1 and protein phosphatase-2A is correlated with an increase in nNOS activity (66, 67). Accordingly, we described an original mode of activation of nNOS by dephosphorylation of tyrosyl residues (39, 41). In the present work, we observed that RC-160 treatment of SST5-expressing cells resulted in transient phosphorylation of nNOS at tyrosyl residues and consequent nNOS inhibition. The kinetics of phosphorylation were in accordance with RC-160-induced SST5-nNOS complex formation and the RC-160 inhibitory effect on nNOS activity. These results confirmed the critical role of tyrosyl phosphorylation in controlling NOS activity.

Like other GPCRs, the SST5 somatostatin receptor does not harbor intrinsic tyrosine kinase activity. Recruitment and activi-
FIGURE 8. The SST5-mediated anti-proliferative action initiated by p60\textsuperscript{src}-dependent nNOS phosphorylation and inactivation is effective in pancreatic endocrine tumor cells. A and B, pancreatic neuroendocrine tumor-derived BON cells were cultured until they were subconfluent. 24 h later, cells were or were not incubated for 1 min with 10 nM RC-160 in the absence of serum. SST5 (A) and nNOS (B) were immunoprecipitated (i.p.) before sequential immunoblotting with anti-p60\textsuperscript{src}, anti-phosphotyrosine (P-Tyr), anti-nNOS, and anti-SST5 antibodies. Results are representative of two immunoblottings, each performed after different sets of RC-160 treatments. C, BON cells were plated for 24 h in 8-chamber slides at 50 \times 10^3 cells/chamber in DMEM containing 10% FCS. Following overnight serum starvation, cells were washed twice with serum-free medium before treatment for 30 min with 10 nM RC-160. Real-time fluorescent cell-associated NOS activity was monitored using an NOS fluorometric detection system with an LSM510 confocal microscope at the times indicated following the manufacturer’s instructions. Results are representative of two real-time NOS activity assays, each performed after different sets of RC-160 treatments. D, BON cells were plated for 24 h in DMEM containing 10% FCS. Following overnight serum starvation, cells were treated in triplicate for 24 h with 10% FCS-containing medium in the presence or absence of RC-160 (10 nM) or the nNOS inhibitor \alpha-guanidinoglutaric acid (GGA; 0.1 \muM). Cell viability was measured at 490 nm using the CellTiter 96 AQueous One Solution cell proliferation assay following the manufacturer’s instructions. Results are the mean ± S.E. of three separate experiments performed in triplicate. **, p < 0.01; ns, not significant.
Regulation of nNOS Activity by Somatostatin Analogs

Tyrosine phosphorylation of Src family kinases are known to play a role in GPCR-mediated signaling pathways (51, 68–73). Src has been recently described to be positively coupled to the SST1 and SST2 somatostatin receptor subtypes (10, 74–76). The data presented herein clearly demonstrate that p60src is involved in SST5-mediated inhibition of nNOS activity and cell proliferation. We first demonstrated that transfecting CHO/SST5 cells with the catalytically inactive p60src mutant abrogated the RC-160 anti-proliferative effect. We next demonstrated that p60src specifically interacted with the SST5 somatostatin receptor and that the somatostatin analog RC-160 strongly induced p60src activation. The participation of p60src in SST5 receptor-mediated activation of the N-methyl-d-aspartate receptor in hippocampal noradrenergic nerve endings has been reported recently (77). The present study provided, to our knowledge, the first demonstration of the activation of p60src by the SST5 somatostatin receptor. Several mechanisms have been shown to mediate p60src activation by GPCRs (50, 52, 78). Recently, we (10) and others (79, 80) demonstrated that β-arrestin, which activates p60src by a G protein βγ-subunit-dependent mechanism (for review, see Ref. 71), can be recruited by activated SST2 receptors. We obtained preliminary results suggesting that expression of a G protein βγ-subunit-sequestering agent might inhibit RC-160-induced binding of p60src to SST5 and subsequent p60src activation. Thus, one possibility is that, in SST5 signaling, G protein βγ-subunits are critical for p60src recruitment and subsequent activation.

p60src tyrosine kinase activation by SST5 occurred concomitantly with SST5-mediated nNOS hyperphosphorylation. These observations suggest that SST5-dependent activation of p60src may initiate a signaling cascade leading to nNOS inactivation following tyrosine phosphorylation. Using the p60src mutant to disrupt SST5 signaling, we demonstrated that nNOS was resistant to phosphorylation and inactivation by somatostatin. In addition, we demonstrated that p60src-associated constitutively with nNOS in CHO/SST5 cells and that nNOS-p60src complex formation was up-regulated by somatostatin binding to SST5. Using recombinant proteins, we further confirmed that p60src directly phosphorylated nNOS monomers at tyrosyl residues. Such phosphorylation prevented nNOS dimerization, indicating that this particular post-translational modification of nNOS is as critical as BH4 and the prostatic heme group for dimer formation and stabilization. Interestingly, recombinant p60src was unable to either phosphorylate or induce monomerization of nNOS homodimers. Thus, SST5-activated p60src prevents dimerization of nNOS rather than inducing monomerization of the enzyme. This is in agreement with previous studies describing nNOS dimers as extremely stable (46, 81). Next, we used multiple approaches to determine the tyrosine(s) that constitute the p60src-binding site of nNOS. Using surface plasmon resonance, we demonstrated a direct interaction of the p60src SH2 domain with nNOS phosphorylated Tyr604. Although that experiment showed that a phosphopeptide containing Tyr604 is a good ligand for the p60src SH2 domain, how this tyrosine is actually phosphorylated in nNOS is currently under investigation. Mutation analysis of this residue led to decreased nNOS-p60src complex formation upon SST5 activation. As a consequence, RC-160 was inefficient in inhibiting NOS activity in SST5-expressing cells transfected with mutant nNOS. These results are consistent with a central role for p60src kinase in nNOS regulation following SST5 activation in CHO/SST5 cells. Interaction of NOS with heterologous proteins has recently emerged as a mechanism by which the activity, spatial distribution, and proximity of NOS isoforms to regulatory proteins and intended targets are governed. An increasingly wide array of proteins, ranging from scaffolding proteins to membrane receptors, have been shown to function as NOS-binding partners. PDZ domains of nNOS bring the enzyme in proximity to the N-methyl-d-aspartate receptor (for review, see Ref. 82) and force its interaction with postsynaptic density proteins PSD-95 and PSD-93 (83), α-synuclein (84), and the protein chaperone CAPON (85). Other proteins such as the protein inhibitor of NOS PIN and the dynamin light chain dissociate the active NOS homodimer (86). We demonstrated previously that protein-tyrosine phosphatases SHP-1 and SHP-2 can associate with nNOS (39, 41). Formation of nNOS-Hsp90 heterocomplexes resulting in enhanced NO formation has also been reported (87). The interaction of nNOS with caveolin-3 in skeletal muscle has been described (88). The present study provides, to our knowledge, the first demonstration of the direct interaction between the protein-tyrosine kinase p60src and the nNOS isoform.

Somatostatin and somatostatin analogs are well characterized inhibitors of pancreatic neuroendocrine tumor cell secretion and growth in vivo and in vivo (55–59). Thus, we investigated whether the mechanism we proposed was effective in tumor cells. We demonstrated that SST5 associated with nNOS and p60src in resting BON cells (derived from human pancreatic carcinoid). RC-160 treatment promoted a rapid increase in SST5-nNOS and SST5-p60src complexes associated with the phosphorylation of nNOS at tyrosyl residues, leading to nNOS inactivation and subsequent inhibition of cell proliferation. The SST5 somatostatin receptor subtype is expressed in various tissues, including the central and peripheral nervous systems and the gastrointestinal tract, and in inflammatory and immune cells. In addition, tumors originating from brain, pancreas, prostate, lung, kidney, breast, and neuroendocrine tissues express SST5, with preferential expression in growth hormone-secreting pituitary adenomas (89). nNOS has been detected in tumors originating from brain, pancreas, prostate, lung, kidney, breast, and neuroendocrine tissues express SST5, with preferential expression in growth hormone-secreting pituitary adenomas (89). nNOS has been detected in different tissues, including brain, skeletal muscle, intestine, and pancreas, where SST5 expression occurs (90, 91). It would therefore be of interest to investigate whether control of NO production by SST5 mediates other cellular functions in addition to control of cell proliferation.

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