Effect of Somatostatin on Nitric Oxide Production in Human Retinal Pigment Epithelium Cell Cultures

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PURPOSE. To investigate the presence of somatostatin and its receptors (sst1–5 receptors) and their possible involvement in the regulation of nitric oxide (NO) production in human RPE cell cultures.

METHODS. Human RPE cells (D407) were used for all studies performed. Somatostatin levels were detected by radioimmunoassay, and RT-PCR and immunocytochemistry studies were performed to identify the somatostatin receptors (sst1–sst5). Radioligand binding assays were also performed examining the ability of certain somatostatin ligands (sst1, sst2, sst5) to compete for [125I]Tyr11 somatostatin binding. The presence of NO synthase in the cultures was assayed with NADPH-diaphorase cytochemistry, and RT-PCR, and NO levels were assessed by examining the production of its stable metabolites NO2− and NO3− (NOx).

RESULTS. SRIF was detected in a concentration of 0.56 ± 0.13 picomoles/mg protein. sst1, sst2, and sst5 mRNAs were detected, yet only sst2m and sst5 immunoreactivity was observed in human RPE cell cultures. sst1 and sst5 but not sst2 selective ligands displaced the specific [125I]Tyr11 somatostatin binding to RPE cell membranes. NADPH-diaphorase stain and iNOS mRNA were detected. SRIF and the sst2 selective analogue MK678 increased the levels of NOx in a concentration-dependent manner. This increase was blocked by the sst antagonist CYN-154806 (Ac-INO2-Phe-c(cGys-Tyr-dTrp-Lys-Cys)-dTyr-NH2).

CONCLUSIONS. These results demonstrate the presence of somatostatin and its receptors sst1, sst2m, and sst5 in human RPE cells and suggest an autocrine or paracrine role for somatostatin. Somatostatin’s ability to regulate NO production, by activating sst2 receptors, provides a functional role of somatostatin in the RPE. (Invest Ophtalmol Vis Sci. 2004;45:1499–1506) DOI:10.1167/iovs.03-0835

The neuropeptide somatostatin (somatotropin release inhibitory factor, SRIF) mediates a diverse number of physiological actions in the peripheral and central nervous system.1,2

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Five SRIF receptor subtypes have been cloned, namely sst1–5 and are responsible for SRIF’s actions. The sst2 receptor has been demonstrated to exist in mice, rats, and humans as two splice variants, sst2A and sst2B.5–7

In the eye, SRIF was initially detected in the retina in amacrine, ganglion, and interplexiform cells and is believed to function as a neurotransmitter, neuromodulator, or trophic factor.8–11 These actions of SRIF are mediated by specific G-protein–coupled receptors, as substantiated by pharmacological12,13 and reverse transcription–polymerase chain reaction (RT-PCR) studies.14 More recent studies employing immunohistochemistry techniques resulted in the identification and localization of the receptor subtypes in retinal cells of different species (for a review see Ref. 15). The colocalization of sst2A and sst2B receptors with NADPH-diaphorase in rod bipolar and photoreceptors cells, respectively, was reported recently,16 introducing for the first time a possible role of SRIF in the regulation of nitric oxide (NO) production in the retina.

RPE is a monolayer of cells situated between the neuroretina and the choroid. It plays an important role in the control of outer retinal homeostasis, in the maintenance of blood-retina barrier integrity,17 and in the regulation of subretinal neovascularization.18 The sst2A receptor subtype has been detected in RPE of normal control human eyes and at different stages of age-related maculopathy.19 In a recent review, van Hagen et al.19 reported the localization of sst1 and sst2A immunoreactivity in human RPE, and sst2A expression in primary human RPE cultures. The presence of sst1 and sst3 (mRNA, immunoreactivity) was also reported in cultured RPE cells.20 Recent studies performed in our laboratory have shown the presence of sst1 immunoreactivity in rat RPE, where it is colocalized with NADPH-diaphorase.21

RPE cells have been shown to produce NO in response to a number of cytokines,22 and it has been suggested that RPE-derived NO may be involved in the maintenance of tight junction integrity.23 The effect of NO on tight junctions was studied in cultured rat RPE, by examining its actions on trans-epithelial electrical resistance (TER) and passive permeation of [3H]insulin across confluent cells. These measurements provided information on the function of tight junctions. NO donors increased TER and the transcellular fluxes of [3H]insulin across confluent cells.

To aid in our understanding of the role of SRIF in RPE physiology, it is important to elucidate the function of its receptors. To this end, the present study investigated the presence of SRIF and its receptor subtypes (sst1–5) and their possible involvement in the regulation of NO production, in a human RPE cell line.

Materials and Methods

Cell Culture

Human RPE cells (D407 cell line23) were grown in DMEM without phenol red containing 5% fetal bovine serum (Gibco BRL, UK), 1%

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Radiolabelling was done using [35S]methionine as standard and radiolabel tracer, respectively. Tin-14 (15,000 cpm; 2,000 Ci/mmol; Amersham, Amersham, UK) was used as standard.

It was then reverse transcribed in a reaction containing 1 μg of RNA, dT(12-18) primers (Amersham Pharmacia Biotech Inc, Piscataway, NJ) It was then reverse transcribed in a reaction containing 1 μg of RNA, dT(12-18) primers (Amersham Pharmacia Biotech Inc, Piscataway, NJ). The reverse transcription was performed on total RNA from human RPE cells using the reverse transcription kit (Promega, Madison, WI).

Denatured at 70°C according to the manufacturer's instructions. Thereafter, samples were stored at -80°C until further use. The lyophilized samples were homogenized in radiomimunooassay buffer containing, 0.1 M sodium phosphate (pH 7.4), 0.15 M sodium chloride, 0.1% gelatin and 0.05% sodium azide. For the determination of SRIF levels, a rabbit antiserum raised against ovalbumin coupled SRIF-14 was used (1:15,000) according to Sperk and Widmann.

Reverse transcription

Reverse transcription–PCR

Reverse transcription–PCR was performed on total RNA from human RPE cells, as previously described by Jordan et al.27 Total RNA was extracted from RPE cells into TRIzol (Invitrogen, Carlsbad, CA) and one microgram of RNA was DNase treated with DNase I (Invitrogen), according to the manufacturer’s instructions. Thereafter, samples were denatured at 70°C for 10 minutes in the presence of 5 mM oligo(dT)12-18 primers (Amersham Pharmacia Biotech Inc, Piscataway, NJ). It was then reverse transcribed in a final 21-μL volume with 10 U/μL (Superscript II; Invitrogen), 1× RT buffer, 0.5 mM deoxyribonucleotide triphosphates (dNTPs; Roche Diagnostics, Mannheim, Germany), 5 mM dithiothreitol (DTT), and 2.5 U/μL RNasin (Promega Corp, Southampton, UK) at 42°C for 60 minutes. One-microliter aliquots of cDNA were PCR amplified in a 25-μL reaction, containing 1× PCR buffer, 0.2 mM dNTPs, 0.5 mM sense and antisense primers, and 0.05 U/μL Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany). The oligonucleotide sequence, annealing temperatures, cycles, and product size for each gene-specific primer pair used are shown in Tables 1 and 2. The primers were synthesized and supplied by MWG (Ebersberg, Germany). The conditions for amplification were 5 minutes at 94°C, X cycles of 30 seconds at 94°C, 30 seconds at 60°C, 30 seconds at 72°C; followed by an extension for 7 minutes at 72°C. To control for genomic contamination, an identical parallel PCR reaction was performed containing starting material that had not been reverse transcribed. PCR products were resolved by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. The RT-PCR studies were performed twice.

**Table 1. Primer Sequences Used for the RT-PCR Studies**

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<th>Gene</th>
<th>UniSTS Code</th>
<th>GenBank or RHdb Code</th>
<th>Primers</th>
<th>Tm (°C)</th>
<th>Product Size (bp)</th>
<th>Cycles</th>
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<td>109142</td>
<td>G49387</td>
<td>F GGTGGCTTTTAGGATGCGAAG</td>
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<td>161</td>
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<td>145</td>
<td>40</td>
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</table>

F, forward; R, reverse.

| Table 2. Primer Sequences Used for the NOS RT-PCR Studies |
|----------------|----------------|----------------|
| GenBank or RHdb Code |
| Primers | Tm (°C) | Product Size (bp) | Cycles |
|----------------|----------------|----------------|
| β-Actin | 109142 | G49387 | F GGTGGCTTTTAGGATGCGAAG | 62.9 | 161 | 30 |
| R ACTGGACCGGTAAGGTGACAG | 63.6 | 555 | 35 |
| iNOS | 84524 | RH79885 | F ACA GGA GGG GTT AAA GCT GC | 60.5 | 232 | 35 |
| R TTG TCT CCA AGG GAC CAG G | 56.7 | | |
| nNOS | 11632 | L02881 | F AGACACAGCCATCACAGGG | 59.8 | | |
| R TGCTTGGCATGATTTCCT | 59.4 | 121 | 35 |
| eNOS* | | | F AAT CCT GTA TGG CTC CGA GA | | |
| R GGG ACA CCA GCT CAT ACT CA | | 121 | 35 |

F, forward; R, reverse.

* Primers were designed by our team.
SRIF sst₂ Receptor Regulation of NO Production

Determination of NO Stable Decomposition Products NO₂⁻ and NO₃⁻ in Human RPE Cultures

The spectrophotometric measurement of the stable decomposition products NO₂⁻ and NO₃⁻ of NO has been used, by many investigators, to determine NO levels indirectly. The protocol used is a slight modification of that of Grisham et al.³⁰

D407 cells seeded in 24-well plates, cultured in serum-free medium for 24 hours, and incubated for 20 minutes in the presence or absence of SRIF-14 (Bachem Bioscience) or SRIF receptor specific analogues CH-275 (sst₁), MK678 (sst₂), L796778 (sst₃), L809877 (sst₄), and L817818 (sst₅), in concentrations ranging from 10⁻¹⁰ to 10⁻⁷ M (three to six replicates per treatment). To examine the selectivity of the sst₂ effect, RPE cells were incubated with SRIF-14 (1 nM) or MK678 (1 nM) in the presence or absence of the sst₂ antagonist CYN-154806 (100 nM).³²

The supernatants of the RPE cultures were incubated for 50 minutes at 37°C in the presence of 0.2 U/mL Aspergillus nitrite reductase, 50 mM HEPES buffer, 5 mM NADPH, 1 mM nitro blue tetrazolium, 10 mM -napthyl-ethylene-diamide, and 5 M pyruvic acid (sodium salt, type II), in a total volume of 300 μL, for the oxidation of any unreacted NADPH. Finally, an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-naphthyl-ethylene-diamide, 2.5% phosphoric acid) was added to each tube, and the samples were measured spectrophotometrically at 543 nm with an ELISA reader. All chemicals were purchased from Sigma-Aldrich (Dingeshofen, Germany), unless indicated otherwise.

Statistics

The mean ± SEM for each group was calculated (experiments were performed four to eight times, see figure legends). An analysis of variance was preferred on computer (Prism, ver. 2.01; GraphPad, San Diego, CA) to detect statistically significant differences among the groups.

Results

Human RPE cell cultures provided a good medium to examine the presence of SRIF in the RPE. By radioimmunoassay, SRIF levels were measured and found to be 0.56 ± 0.13 picomoles/mg protein (n = 6).

RT-PCR analysis using primers based on human sequences (Table 1) detected mainly the presence of sst₁ and sst₂ mRNA and low but detectable levels of sst₃ mRNA. sst₃ and sst₄ mRNAs were not detected (Fig. 1).

Antibodies raised against carboxyl terminal fragments of human sst₁ to sst₄ were used to assess the presence of the SRIF receptors in the human RPE cell cultures. Although sst₁ mRNA and contrast adjustment of images were processed with the use of image-analysis software (Photoshop, ver 5.0; Adobe Systems, Mountain View, CA).

Radioligand-Binding Studies

[¹²⁵I]Tyr₁₁ somatostatin binding (120 pM; 2000 Ci/mmol) was examined as described in Vasilaki et al.¹³ Cell membranes (70 μg) were incubated with radioligand for 90 minutes at 25°C and compared with the 100-kb pair ladder in the first lane.

382-391; hsst₂A; FRNNKRKK; 348-356 aa; hsst₂B, 384-393; hsst₃, 366-388; hsst₄, and 345-364; hsst₅.

They have been characterized and used to localize the different SRIF receptor subtypes (sst₁⁻⁵) in human tumors.³⁶ The selectivity of the human sst₃ and sst₄ immunoreactivity was examined in cells incubated with antibodies that were preadsorbed for 1 hour with synthetic peptides (10 μg/mL) corresponding to the carboxyl terminal sequences of hsst₃ (residues 348-356) and hsst₅ QEATRPRTAAANGLMQTSKL (residues 345-364) receptors, respectively. All antibodies and antigens were obtained from Stefan Schulz (Otto-von-Guericke University, Magdeburg, Germany). The immunocytochemistry studies were performed four to six times.

NADPH-Diaphorase Cytochemistry

The NADPH-diaphorase histochemical technique has been used as a nuclear marker (C). Scale bar, 20 μm.

FIGURE 2. The sst₂₈ immunoreactivity in human RPE cells. The sst₂₈ immunostain is present in the nucleus of D407 human RPE cells. Control sections incubated with the sst₂₈ antibody preblocked with antigen (hsst₂₈, FRNNKRKK, 348-356 aa; 10 μg/mL) show no immunoreactivity. DAPI (4 μg/mL) stain was used as a nuclear marker (C). Scale bar, 20 μm.
was detected, as described earlier, no sst1 immunoreactivity was observed in the RPE cells (data not shown). The sst2B immunoreactivity was observed mostly in the perinuclear region and nucleus of individual RPE cells (Fig. 2, left panel). Preblocking of the antibody with the respective antigen provided evidence for the specificity of the signal (Fig. 2, middle panel). DAPI stained the RPE cells, and the image was very similar to that observed with the sst2B antibody, thus substantiating the nuclear localization of the receptor (Fig. 2, right panel). The SRIF receptor subtypes sst2A, sst3, and sst4 were not detected (data not shown).

Radioligand binding assays performed on RPE cell membranes suggested the presence of sst1 and sst5, but not sst2, on the cell membranes. The sst1 and sst5 selective analogues CH275 (1 μM) and L-817818 (1 μM), respectively, displaced 76% ± 13% and 81% ± 10% of specific 125I-Tyr11 somatostatin binding, while the sst2-selective analogue MK678 (1 μM) had no effect.

NADPH-diaphorase stain was also observed in human RPE cells and was localized primarily in the cytoplasm, as observed by light microscopy (Fig. 4). RT-PCR analysis using primers based on human sequences (Table 1) detected only iNOS (Fig. 5). The presence of the SRIF receptors, NADPH-diaphorase and iNOS in the RPE cells suggested a possible role of SRIF in the regulation of NO production. To assess this directly, SRIF and selective SRIF receptor agonists were applied to the cells and the production of its stable NOx− metabolites assessed (Fig. 6).

Basal levels of NOx− were found to be 11.8 ± 0.9 μM/mg protein (n = 9). SRIF and the sst2-selective analogue MK678 increased the production of NOx− in a concentration-dependent manner (Figs. 6A, 6B). Selective analogues for sst1 and sst3 had no statistically significant effect on the NOx− levels (Figs. 6C, 6D) nor did analogues for the sst4 (data not shown).

To assess further the pharmacological significance of the above data, the ability of the selective sst2 antagonist CYN154806 (100 nM) to block the SRIF- and MK678-induced increase of NOx− was examined. Indeed, CYN154806 was able to block the SRIF- and MK678-induced increase of NOx− (Fig. 7A, 7B), whereas it had no statistically significant effect on NOx− levels when administered alone (Fig. 7C).

**DISCUSSION**

SRIF was found to be present in human RPE cells, in agreement with the findings of van Hagen et al., who observed SRIF-14 mRNA expression in primary human RPE cultures. Using antibodies raised against human fragments of the receptors, only the sst2B and sst5 receptors were localized in individual cells of the RPE cultures. The sst2B immunoreactivity was localized primarily in the perinuclear region and nucleus of individual RPE cells, whereas sst5 immunostain was localized in the cytoplasmic granular compartment.

Klisovic et al. reported the presence of sst4 on cell membranes, but also a significant amount in the cytoplasm, perinuclear region, and nucleus of RPE cells in culture. In the present

![Figure 3](image-url)  
**FIGURE 3.** The sst5 immunoreactivity in human RPE cells. The sst5 immunostain is present in the granular cytoplasmic compartment of D407 human RPE cells. Control sections incubated with the sst5 antibody preblocked with antigen (hsst5; QEQAT-RPRTAAANGMQTSKL; 345-364 aa; 10 μg/mL) show no immunoreactivity. Scale bar, 20 μm.

![Figure 4](image-url)  
**FIGURE 4.** NADPH-diaphorase cytochemistry in human RPE cells. NADPH-diaphorase staining is present in the cytoplasm of D407 human RPE cells. Scale bar, 50 μm.
study, although sst1 mRNA was detected by RT-PCR, no immunoreactivity was observed in the RPE cells. However, the sst1-selective analogue CH275 was able to displace the specific \([^{125}\text{I}]\text{Tyr}^{11}\) somatostatin binding, suggesting the presence of the sst1 receptor in RPE cell membranes. Immunoreactivity for sst1 was observed in rat RPE\(^{21}\) and in human RPE tissue\(^{19}\); thus, the discrepancy observed in the immunocytochemistry data of the present study may be due to technical differences (e.g., different RPE cell lines, D407 present study versus ATCC-2303,\(^{20}\) antibodies used and/or species diversity).

sst2 mRNA was present in RPE cells, and this was reflected by the detection of sst2B immunoreactivity. The intracellular localization of sst2B immunoreactivity in the perinuclear and nuclear area of individual cells shown in the present study was also observed by Klisovic et al.\(^{20}\) for the sst2 receptor. These investigators used an antibody that was raised against the N-terminal 45 amino acids that are common in both sst2A and sst2B. Therefore, the observed sst2 immunoreactivity may, at least in part, be due to the presence of sst2B receptors. The intracellular localization of the receptor suggests that soma-
**A** somatostatin (1 nM; FIGURE 7). **B** Effect of the sst₂ antagonist CYN-154806 on somatostatin and MK678 induced release of stable NO decomposition products NOₓ. The sst₂ antagonist CYN-154806 (100 nM) blocked the somatostatin (1 nM; A) and the sst₂-selective analogue MK678 (1 nM; B) increase of NOₓ production. *P < 0.05 agonist versus control, #P < 0.05 agonist + sst₂ antagonist versus agonist, n = 4, paired t-test. The antagonist alone had no statistically significant effect (C).

tostatin found in the RPE cells is able to regulate the membrane receptors and promote their internalization. In agreement with this hypothesis, Reubi et al.\(^{33}\) presented evidence showing that tumor-produced somatostatin can act in an autocrine fashion to internalize the sst₂A receptor, whereas Dournaud et al.\(^{33}\) have shown that high levels of local endogenous somatostatin in rat brain results in the internalization of sst₂A receptors. Furthermore, Koenig et al.\(^{35}\) have shown that somatostatin agonists, as well as the sst₂ receptor, cycle continuously between the cell surface and the intracellular compartments. The significance of the present findings and those of Klisovic et al.\(^{20}\) regarding the localization of the sst₁ and sst₂/sst₂B receptors in the perinuclear and nuclear regions should be investigated further.

To our knowledge, this is the first report showing the localization of sst₂ receptors in the RPE (cells or tissue). Mori et al.\(^{1}\) had reported the presence of sst₄ mRNA in a mixture of rat retina-free posterior eye segment that included the RPE, choroid, sclera, and optic nerve. In an elegant study, Stroh et al.\(^{36}\) examined the intracellular dynamics of sst₄ receptors in transfected COS-7 cells, their internalization, and recycling. These investigators presented the kinetics of sst₄ internalization to the cytoplasm pool in the core of the cell that may represent Golgi stores. The present radioligand binding and immunoreactivity findings suggest that the sst₄ receptor in RPE cells may follow similar patterns of internalization and recycling to the cell membrane.

These findings suggest that SRIF may differentially influence RPE physiology by activating different receptor subtypes. The possible involvement of SRIF in the regulation of NO production in the RPE, as was previously shown in the retina,\(^{16,21}\) was examined. NADPH-diaphorase cytostain was evident, whereas RT-PCR studies suggest the presence of iNOS in agreement with Faure et al.\(^{37}\) Indeed, SRIF increased the production of NOₓ in a concentration-dependent manner at physiological concentrations (10⁻¹⁰ and 10⁻⁹ M). This effect was mediated through activation of the sst₂ receptor, as observed in the retina.\(^{21}\) Multiple studies have presented evidence of somatostatin internalization. Somatostatin and other agonists were found to accumulate inside the cytoplasm,\(^{34}\) in the center of the cells in close proximity to the nucleus.\(^{36}\) Internalization, nuclear translocation, and DNA binding were also observed.\(^{38}\) These data present supportive evidence and a means to explain how the sst₂B receptor found intracellularly in the RPE cells can be activated and regulate NO production. However, the actual mechanisms involved should be further investigated. The sst₂B receptor subtype is characterized by a shorter C-terminal tail (23 amino acids shorter), compared with the sst₂A subtype,\(^{5,6}\) and thus lacks the phosphorylation sites that are needed for receptor desensitization and internalization. However, Beaumont et al.\(^{39}\) have presented evidence showing that the sst₂B receptor also internalizes, and this action leads to its desensitization.

The presence of somatostatin and its receptors in the RPE cells suggests an autocrine role for somatostatin. As mentioned earlier, somatostatin present in the RPE cells may bind to sst₁, sst₂B, and sst₄ receptors and modulate their activities (e.g., internalization for sst₂A and sst₂). However, this does not exclude the possibility that somatostatin synthesized in the RPE may activate somatostatin receptors in the photoreceptor layer,\(^{11,16,40}\) thus acting in a paracrine fashion and influencing RPE-retinal interactions.

The use of octreotide in cystoid macular edema complements the present findings and suggests a possible role of SRIF in the regulation of ion/water transport systems located in the RPE.\(^{41}\) The ability of SRIF at physiological concentrations of 0.1 and 1.0 nM to influence the production of NO in the RPE cultures suggests that SRIF produced by these cells may regulate NO function in vivo.
NO secretion has been shown to increase cytokine stimulation of the RPE, and is believed to play a role in the blood-retina barrier and in the development of immune and inflammatory responses in the eye. SRIF and its analogues have been shown to have a suppressive effect on the immune response and to have antiproliferative effects on retinal endothelial cells.

Long-acting somatostatin analogues (octreotide and lanreotide) have recently been used for the treatment of retinopathies. The present results in conjunction with published data suggest that somatostatin agonists through activation of sst2 receptors can increase NO levels and help restore and maintain the integrity of the blood retinal barrier and improve visual acuity.

In conclusion, the results from the present study demonstrate for the first time that SRIF is able to regulate NO production in the RPE by activating sst2 receptors. The actual physiological significance of this action, as well as of the involvement of sst1, sst3, or sst5 in RPE physiology warrants further investigation.

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