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 (sst_{1-5} 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 (sst_1-sst_5). Radioligand binding assays were also performed examining the ability of certain somatostatin ligands (sst_1, sst_2, sst_5) to compete for [^{125}I]Tyr^{11} 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 NO_2^- and NO_3^- (NO_x^-).

Results. SRIF was detected in a concentration of 0.56 ± 0.13 picomoles/mg protein. sst_1, sst_2, and sst_5 mRNAs were detected, yet only sst_{2B} and sst_5 immunoreactivity was observed in human RPE cell cultures. sst_1- and sst_5- but not sst_2-selective ligands displaced the specific [^{125}I]Tyr^{11} somatostatin binding to RPE cell membranes. NADPH-diaphorase stain and iNOS mRNA were detected. SRIF and the sst_2-selective analogue MK678 increased the levels of NO_x^- in a concentration-dependent manner. This increase was blocked by the sst_2 antagonist CYN-154806 (Ac-4NO_2-Phe-c(dCys-Tyr-dTrp-Lys-Thr-Cys)-dTyr-NH_2).

Conclusions. These results demonstrate the presence of somatostatin, and its receptors sst_1, sst_{2B}, and sst_5 in human RPE cells and suggest an autocrine or paracrine role for somatostatin. Somatostatin's ability to regulate NO production, by activating sst_2 receptors, provides a functional role of somatostatin in the RPE. (*Invest Ophthalmol 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] Five SRIF receptor subtypes have been cloned, namely sst_{1-5}[3,4] and are responsible for SRIF's actions. The sst_2 receptor has been demonstrated to exist in mice, rats, and humans as two splice variants, sst_{2A} and sst_{2B}.[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 pharmacological[12,13] 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 sst_{2A} and sst_{2B} 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 sst_{2A} receptor subtype has been detected in RPE of normal control human eyes and at different stages of age-related maculopathy.[18] In a recent review, van Hagen et al.[19] reported the localization of sst_1 and sst_{2A} immunoreactivity in human RPE, and sst_{2A} expression in primary human RPE cultures. The presence of sst_1 and sst_2 (mRNA, immunoreactivity) was also reported in cultured RPE cells.[20] Recent studies performed in our laboratory have shown the presence of sst_1 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 transepithelial electrical resistance (TER) and passive permeation of [^{3}H]insulin across confluent cells. These measurements provided information on the function of tight junctions. NO donors increased TER and the transepithelial fluxes of [^{3}H]insulin, suggesting that NO could play an important role in the regulation of blood barrier function.[24]

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 (sst_{1-5}) 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 line[25]) were grown in DMEM without phenol red containing 3% fetal bovine serum (Gibco BRL, UK), 1%

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TABLE 1. Primer Sequences Used for the RT-PCR Studies

Gene	UniSTS Code	GenBank or RHdb Code		Primers	Tm (°C)	Product Size (bp)	Cycles
β-Actin	109142	G49387	F	GGTGGCTTTTAGGATGGCAAG	62.9	161	30
			R	ACTGGACGGTGAAGGTGACAG	63.6		
sst_1	186758	G67495	F	CCACCAACATCTACATCCTA	55.3	555	35
			R	CCACCATCATCACCATTAAG	55.3		
sst_2	186761	G67500	F	CATCTTCTGCCTGACAGTC	56.7	509	35
			R	CCACCACAAAGTCAAACAT	52.4		
sst_3	186764	G67503	F	AGAACGCCCTGTCCTACTGG	61.4	533	40
			R	GTTGACGATGTTGAGCACG	56.7		
sst_4	3081	RH69015	F	AACCTCGTCGTGACCAG	55.2	207	40
			R	AGCAGTGGCATAGTAGTCCAG	59.8		
sst_5	50681	RH66813	F	GCTTCCAGAAGGTTCTGTGC	59.4	145	40
			R	TTGCTGGTCTGCATAAGCC	56.7		

F, forward; R, reverse.

L-glutamine, 0.35% wt/vol glucose glutamine and 1% penicillin/streptomycin (all from Invitrogen-Gibco BRL, Paisley, UK).

Radioimmunoassay

Human RPE cells seeded in 24-well plates were homogenized in 0.5 mL (2 N acetic acid per well), boiled for 10 minutes, sonicated, and kept at −80°C. After at least 24 hours of freezing, the homogenates were centrifuged for 20 minutes at 4°C, and the supernatants were lyophilized and stored at −80°C until further use. The lyophilized samples were homogenized in radioimmunoassay buffer containing, 0.15 M sodium phosphate (pH 7.4), 0.15 M sodium chloride, 0.1% gelatin and 0.02% 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.[26] Synthetic SRIF-14 (Bachem Bioscience, Heidelberg, Germany) and [^{125}I]Tyr^{11} somatostatin-14 (15, 000 cpm; 2,000 Ci/mmol; Amersham, Amersham, UK) were used as standard and radiolabel tracer, respectively.

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 (DDT), 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 56°C to 60°C, and 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.

Immunocytochemical Studies

D407 cells were grown overnight on coverslips and fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (PB) for half an hour at room temperature. After blocking in 0.1 M Tris-HCl buffered saline (TBS; pH 7.4) containing 3.3% normal goat serum for 30 minutes, cells were incubated with rabbit polyclonal primary antibodies raised against human sst_1 to sst_5 (2.5 μg/mL) in 0.1 M TBS containing 0.5% normal goat serum and 0.3% Triton X-100, overnight at room temperature. Subsequently, the sections were washed in TBS and incubated in fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit IgG (1: 150; H+L; Vector Laboratories, Burlingame, CA) secondary antibody for 1.5 hours at room temperature. Finally, cells were rinsed with 0.1 M TBS, mounted with antifade mounting medium (Vector Laboratories), and examined by light microscopy. To visualize nuclear staining, cells were incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 4 μg/mL] in 0.1 M TBS) for 5 minutes.

The polyclonal antibodies used in this study were raised against somatostatin receptor peptide fragments corresponding to residues;

TABLE 2. Primer Sequences Used for the NOS RT-PCR Studies

Gene	UniSTS Code	GenBank or RHdb Code		Primers	Tm (°C)	Product Size (bp)	Cycles
β-Actin	109142	G49387	F	GGTGGCTTTTAGGATGGCAAG	62.9	161	35
			R	ACTGGAACGGTGAAGGTGACAG			
iNOS	84524	RH79885	F	ACA GGA GGG GTT AAA GCT GC	60.5	232	35
			R	TTG TCT CCA AGG GAC CAG G			
nNOS	I1632	L02881	F	AGACACAGCCATCAGACGC	59.8	142	35
			R	TCGGTGGCATGATTTCCT			
eNOS*			F	AAT CCT GTA TGG CTC CGA GA	59.4	121	35
			R	GGG ACA CCA CGT CAT ACT CA			

F, forward; R, reverse.

* Primers were designed by our team.

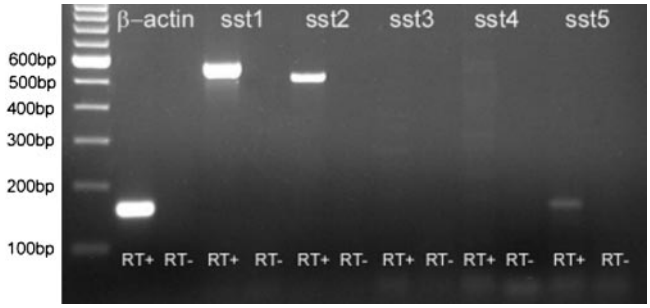

FIGURE 1. RT-PCR studies in human RPE cells. RPE cells express somatostatin receptors sst_1, sst_2, and sst_5. The RT negative control was consistently negative. The gel shows fluorescence of ethidium bromide stained PCR products resolved by electrophoresis. Bands were detected under UV light and compared with the 100-kb pair ladder in the first lane.

382-391; $hsst_1$, 355-369; $hsst_{2A}$, 348-356; $hsst_{2B}$, 384-393; $hsst_3$, 366-388; $hsst_4$, and 345-364; $hsst_5$.

They have been characterized and used to localize the different SRIF receptor subtypes (sst_{1-5}) in human tumors.[28] The selectivity of the human sst_{2B} and sst_5 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 $hsst_{2B}$ FRNNKNRKK (residues 348-356) and $hsst_5$ 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.

Radioligand-Binding Studies

[^{125}I]Tyr^{11} 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 in the absence or presence of CH275 (sst_1, 1 μM), MK678 (sst_2, 1 μM), and L-817818 (sst_5, 1 μM). Specific binding was defined in the presence of somatostatin (1 μM).

NADPH-Diaphorase Cytochemistry

The NADPH-diaphorase histochemical technique has been used as a marker by many investigators to assess the distribution of NO synthase activity in brain and retina.[29] D407 cells were grown overnight on coverslips and fixed with 2% paraformaldehyde in 0.1 M PB for half an hour at room temperature. After rinsing two times for 10 minutes each in PB at room temperature and three times at 10 minutes in 0.1 M Tris-HCl (pH 7.4) at 37°C, cells were incubated in 0.1 M Tris-HCl, containing 0.8 mM β-NADPH, 1 mM nitro blue tetrazolium, 10 mM malic acid and 1.5% Triton X-100 at 37°C for 1.5 hours. Finally, cells were rinsed with 0.1 M Tris-HCl, air-dried, dehydrated, and mounted. The cytochemistry studies were performed four to six times.

Microscopy

Light microscopy images were taken with a (Axioskop with Plan-Neofluar x40/0.75; Carl Zeiss Meditec, Oberkochen, Germany). Light and contrast adjustment of images were processed with the use of image-analysis software (Photoshop, ver 5.0; Adobe Systems, Mountain View, CA).

Determination of NO Stable Decomposition Products NO_2^- and NO_3^- in Human RPE Cultures

The spectrophotometric measurement of the stable decomposition products NO_2^- and NO_3^- 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 (sst_1), MK678 (sst_2), L-796778 (sst_3), L-809087 (sst_4), and L-817818 (sst_5),[31] in concentrations ranging from 10^{-10} to 10^{-5} M (three to six replicates per treatment). To examine the selectivity of the sst_2 effect, RPE cells were incubated with SRIF-14 (1 nM) or MK678 (1 nM) in the presence or absence of the sst_2 antagonist CYN-154806 (100 nM).[32] The culture media were collected, centrifuged for 15 minutes at 12,000 rpm, at 4°C, and the supernatants were kept at −80°C until further use.

The supernatants of the RPE cultures were incubated for 30 minutes at 37°C in the presence of 0.2 U/mL *Aspergillus* nitrate reductase, 50 mM HEPES buffer, 5 μM FAD and 0.1 mM NADPH in a total volume of 200 μL for the reduction of NO_3^- to NO_2^-. Subsequently, the samples were incubated for 10 minutes at 37°C in the presence of 13.5 U/mL lactate dehydrogenase (LDH; bovine muscle) and 9 mM pyruvic acid (sodium salt, type II), in a total volume of 300 μL, for the oxidization of any unreacted NADPH. Finally, an equal volume of Griess reagent (1% sulfanilamide, 0,1% N-napthyl-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 (Diesenhofen, 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 preformed 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 sst_1 and sst_2 mRNA and low but detectable levels of sst_5 mRNA. sst_3 and sst_4 mRNAs were not detected (Fig. 1).

Antibodies raised against carboxyl terminal fragments of human sst_1 to sst_5 were used to assess the presence of the SRIF receptors in the human RPE cell cultures. Although sst_1 mRNA

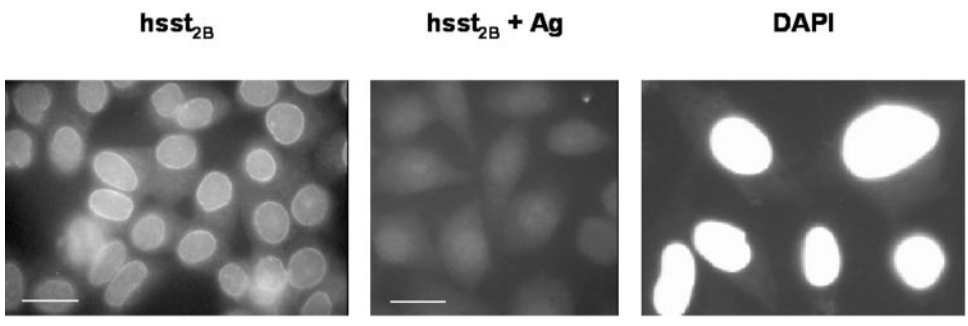

FIGURE 2. The sst_{2B} immunoreactivity in human RPE cells. The sst_{2B} immunostain is present in the nucleus of D407 human RPE cells. Control sections incubated with the sst_{2B} antibody preblocked with antigen ($hsst_{2B}$; FRNNKNRKK; 348-356 aa; 10 μg/mL) show no immunoreactivity. DAPI (4 μg/mL) stain was used as a nuclear marker (C). Scale bar, 20 μm.

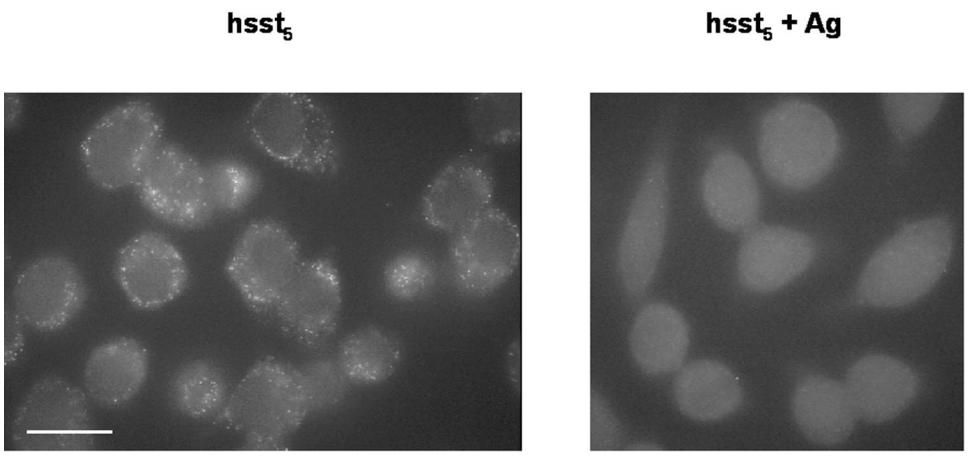

FIGURE 3. The sst_5 immunoreactivity in human RPE cells. The sst_5 immunostain is present in the granular cytoplasmic compartment of D407 human RPE cells. Control sections incubated with the sst_5 antibody preblocked with antigen ($hsst_5$; QEATRPRTAAANGLMQTSKL; 345-364 aa; 10 μg/mL) show no immunoreactivity. Scale bar, 20 μm.

was detected, as described earlier, no sst_1 immunoreactivity was observed in the RPE cells (data not shown). The sst_{2B} 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 sst_{2B} antibody, thus substantiating the nuclear localization of the receptor (Fig. 2, right panel). The sst_5 immunoreactivity appeared to be enriched in a cytoplasmic granular compartment, as shown in Figure 3 (left panel). This signal was specific as ascertained by preblocking of the antibody with the respective antigen (Fig. 3, right panel). The SRIF receptor subtypes sst_{2A}, sst_3, and sst_4 were not detected (data not shown).

Radioligand binding assays performed on RPE cell membranes suggested the presence of sst_1 and sst_5, but not sst_2, on the cell membranes. The sst_1 and sst_5 selective analogues CH275 (1 μM) and L-817818 (1 μM), respectively, displaced 76% $\pm$ 13% and 81% $\pm$ 10% of specific [^{125}I]Tyr^{11} somatostatin binding, while the sst_2-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 NO_x^- metabolites assessed (Fig. 6). Basal levels of NO_x^- were found to be 11.8 $\pm$ 0.9 μM/mg protein (n = 9). SRIF and the sst_2-selective analogue MK678 increased the production of NO_x^- in a concentration-dependent manner (Figs. 6A, 6B). Selective analogues for sst_1 and sst_5 had no statistically significant effect on the NO_x^- levels (Figs. 6C, 6D) nor did analogues for the sst_3 and sst_4 (data not shown).

To assess further the pharmacological significance of the above data, the ability of the selective sst_2 antagonist CYN-154806 (100 nM) to block the SRIF- (1 nM) and MK678- (1 nM) induced increase of NO_x^- levels was examined. Indeed, CYN-154806 was able to block the SRIF- and MK678-induced increase of NO_x^- (Fig. 7A, 7B), whereas it had no statistically significant effect on NO_x^- 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.,[19] who observed SRIF-14 mRNA expression in primary human RPE cultures. Using antibodies raised against human fragments of the receptors, only the sst_{2B} and sst_5 receptors were localized in individual cells of the RPE cultures. The sst_{2B} immunoreactivity was localized primarily in the perinuclear region and nucleus of individual RPE cells, whereas sst_5 immunostain was localized in the cytoplasmic granular compartment.

Klisovic et al.[20] reported the presence of sst_1 on cell membranes, but also a significant amount in the cytoplasm, perinuclear region, and nucleus of RPE cells in culture. In the present

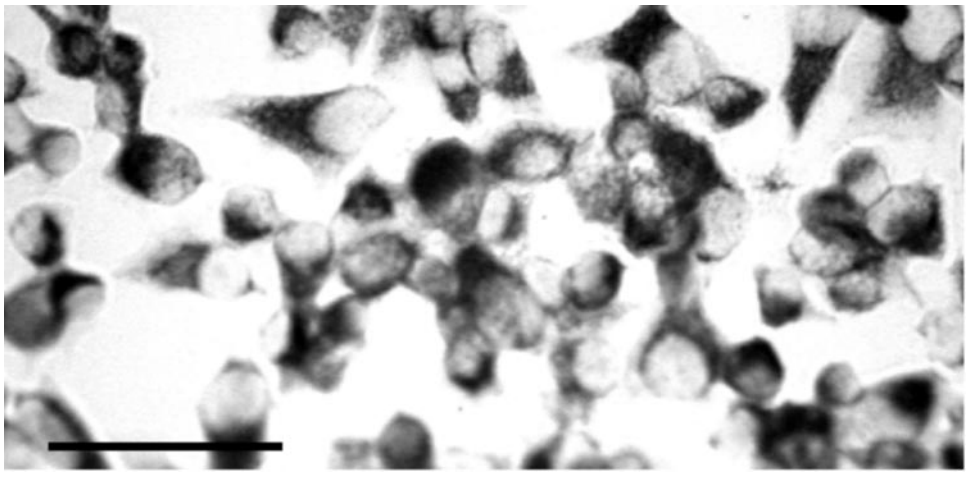

FIGURE 4. NADPH-diaphorase cytochemistry in human RPE cells. NADPH-diaphorase staining is present in the cytoplasm of D407 human RPE cells. Scale bar, 50 μm.

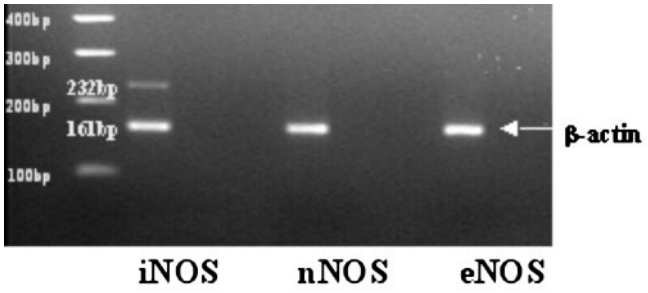

FIGURE 5. RT-PCR (35 cycles) studies in human RPE cells. RPE cells express iNOS but not nNOS or eNOS. The RT negative control was consistently negative. The gel shows fluorescence of ethidium-bromide–stained PCR products resolved by electrophoresis. Bands were detected under UV light and compared with the 100-kb pair ladder in the first lane.

study, although sst_1 mRNA was detected by RT-PCR, no immunoreactivity was observed in the RPE cells. However, the sst_1-selective analogue CH275 was able to displace the specific [^{125}I]Tyr^{11} somatostatin binding, suggesting the presence of the sst_1 receptor in RPE cell membranes. Immunoreactivity for sst_1 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).

sst_2 mRNA was present in RPE cells, and this was reflected by the detection of sst_{2B} immunoreactivity. The intracellular localization of sst_{2B} 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 sst_2 receptor. These investigators used an antibody that was raised against the N-terminal 45 amino acids that are common in both sst_{2A} and sst_{2B}. Therefore, the observed sst_2 immunoreactivity may, at least in part, be due to the presence of sst_{2B} receptors. The intracellular localization of the receptor suggests that soma-

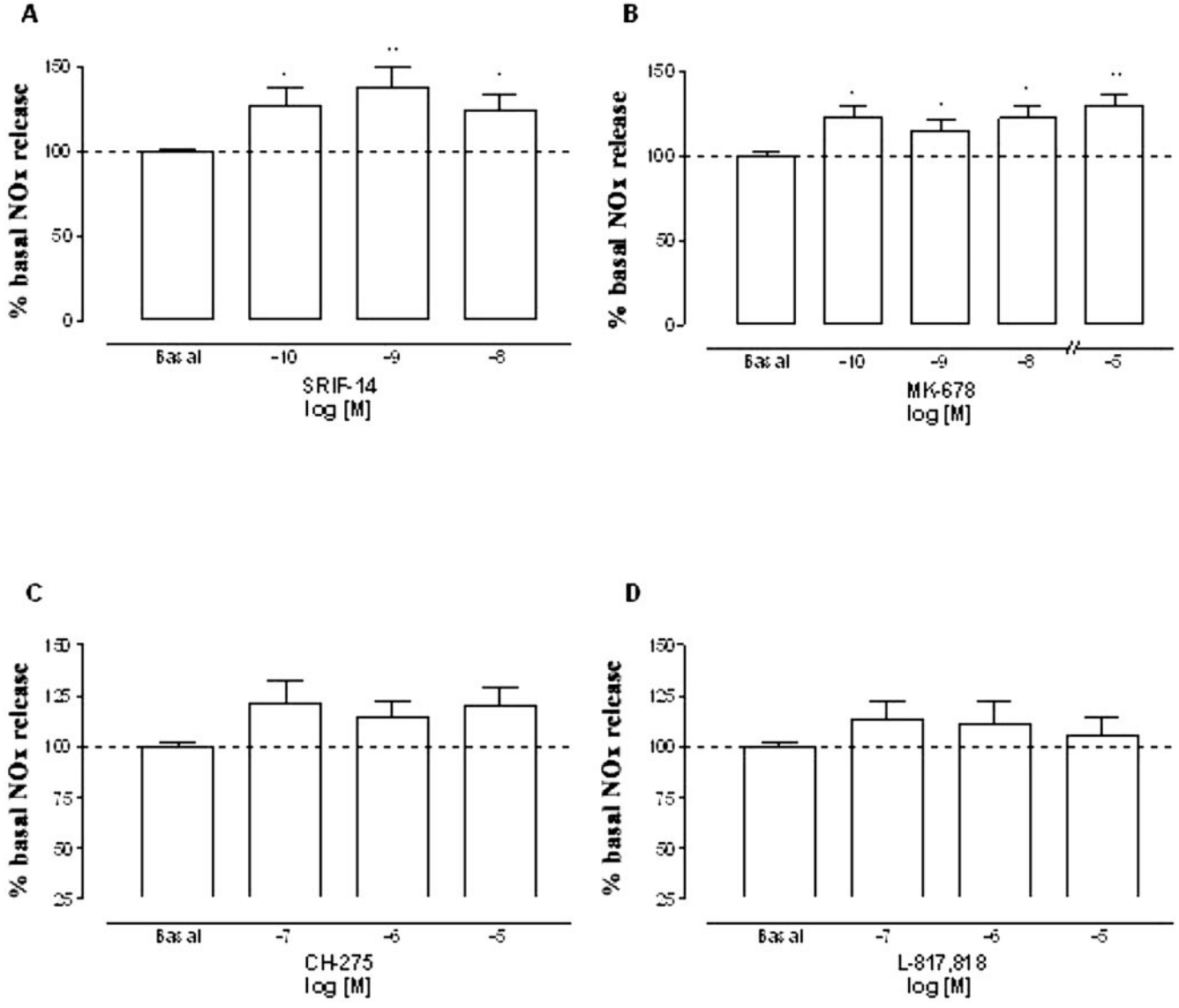

FIGURE 6. Effect of somatostatin and specific analogues on the release of stable NO decomposition products NO_x^- in human RPE cells. Somatostatin (**A**, $n = 7$) and the sst_2-selective analogue MK678 (**B**, $n = 7$) increased NO_x^- release in a concentration-dependent and statistically significant manner. The sst_1, CH-275 (**C**, $n = 7$); and the sst_5, L-817818 (**D**, $n = 8$) selective analogues had no effect. $^*P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$ unpaired *t*-test.

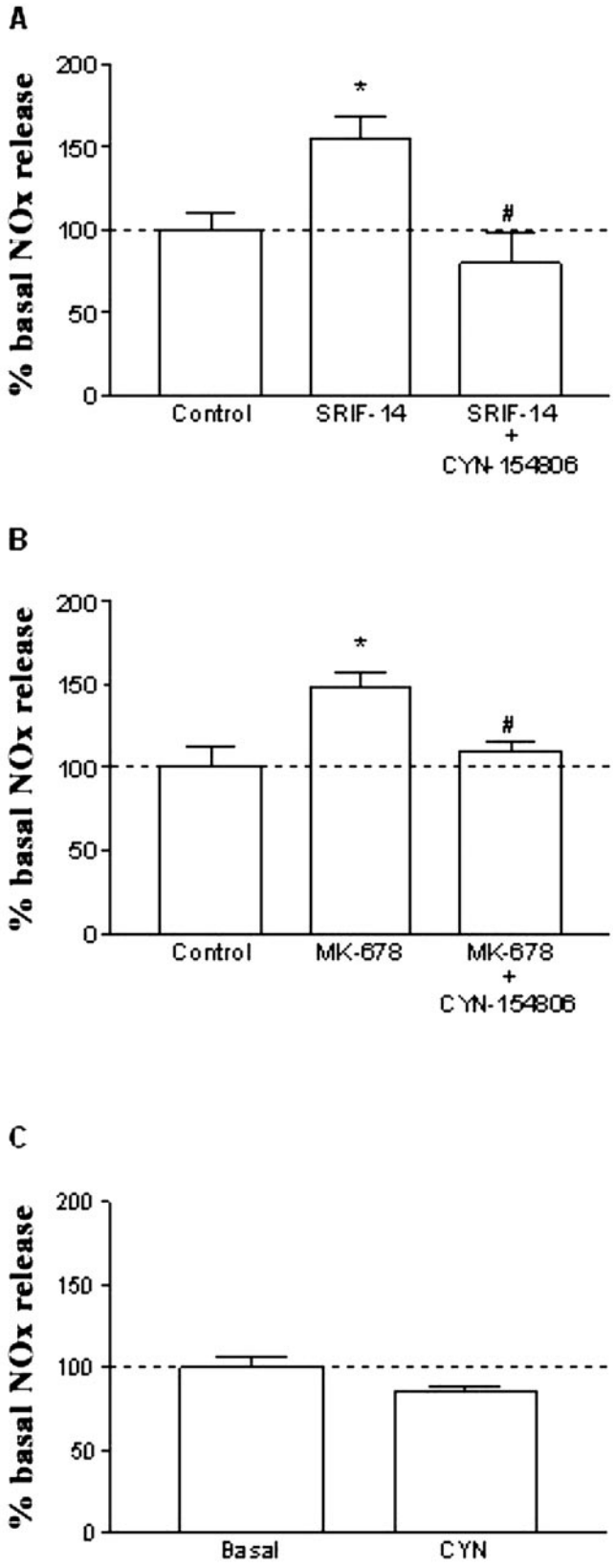

FIGURE 7. Effect of the sst_2 antagonist CYN-154806 on somatostatin and MK678 induced release of stable NO decomposition products NO_x^-. The sst_2 antagonist CYN-154806 (100 nM) blocked the somatostatin (1 nM; **A**) and the sst_2-selective analogue MK678 (1 nM; **B**) increase of NO_x^- production. $^*P < 0.05$ agonist versus control, $^\#P < 0.05$, agonist+sst_2 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 sst_{2A} receptor, whereas Dournaud et al.[34] have shown that high levels of local endogenous somatostatin in rat brain results in the internalization of sst_{2A} receptors. Furthermore, Koening et al.[35] have shown that somatostatin agonists, as well as the sst_2 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 sst_1 and sst_2/sst_{2B} receptors in the perinuclear and nuclear regions should be investigated further.

To our knowledge, this is the first report showing the localization of sst_5 receptors in the RPE (cells or tissue). Mori et al.[14] had reported the presence of sst_5 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 sst_5 receptors in transfected COS-7 cells, their internalization, and recycling. These investigators presented the kinetics of sst_5 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 sst_5 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 NO_x^- in a concentration-dependent manner at physiological concentrations (10^{-10} and 10^{-9} M). This effect was mediated through activation of the sst_2 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 sst_{2B} receptor found intracellularly in the RPE cells can be activated and regulate NO production. However, the actual mechanisms involved should be further investigated. The sst_{2B} receptor subtype is characterized by a shorter C-terminal tail (23 amino acids shorter), compared with the sst_{2A} 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 sst_{2B} 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 sst_1, sst_{2B}, and sst_5 receptors and modulate their activities (e.g., internalization for sst_{2B} and sst_5). 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.[23] SRIF and its analogues have been shown to have a suppressive effect on the immune response[19] and to have antiproliferative effects on retinal endothelium.[42]

Long-acting somatostatin analogues (octreotide and lanreotide) have recently been used for the treatment of retinopathies.[19,43] The present results in conjunction with published data[23] suggest that somatostatin agonists through activation of sst_2 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 sst_{2B} receptors. The actual physiological significance of this action, as well as of the involvement of sst_1, sst_{2B}, or sst_5 in RPE physiology warrant further investigation.

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