6-22-2007

Somatostatin Receptor Subtype 2 is Functionally Expressed in RAW264.7 Cells

Matthew Sung
Seton Hall University

SOMATOSTATIN RECEPTOR SUBYTPE 2 IS FUNCTIONALLY EXPRESSED IN RAW264.7 CELLS

By

MATTHEW SUNG

Submitted in partial fulfillment of the requirements for the
Degree of Master of Science in Biology from the
Department of Biology of Seton Hall University
June 22, 2007

APPROVED BY

MENTOR

DIRECTOR OF GRADUATE STUDIES

CHAIRPERSON, BIOLOGY DEPARTMENT

ACKNOWLEDGEMENTS

I would like to impart my deepest gratitude to the following people:

Dr. Allan Blake, my mentor and thesis advisor, whose oversight and guidance over my Time at Seton Hall University proved to be invaluable. Not only is he a gifted scientist but a well-versed and experienced mentor. It was his lectures and experiences in his laboratory that drove my scientific curiosity and helped me determine what it is about science that I so endear. I pledge my greatest and deepest thanks and appreciation to Dr. Blake for affording me the opportunity to work alongside him.

Dr. Jane Ko, whom I owe much thanks for her insightful discussions about not only my thesis work but graduate school life as a whole and for sitting on my committee.

Dr. Angela Klaus, for sitting on my committee and her advice with various aspects of confocal microscopy.

Jennifer Peek, Kelly Flock, Frances Mae West, Caitlin White, Viren Jadeja, Adriana Gavrilcuic and Alison Bossert, whom are fellow graduate and undergraduate students who assisted me with much of this thesis' work and provided a deep sense of support throughout my tenure at Seton Hall University.

Dr. Carolyn Bentivegna, chairperson of the Department of Biological Sciences at Seton Hall University, for allowing me to serve as a teaching assistant for this department and perform research within its laboratories.

The Department of Biological Sciences Faculty and Staff, who not only provided the best instruction in the classroom but also keeping me on the path throughout my time here with advice and encouragement to continue the fight.

Family and friends, whose love and support cannot be thanked enough.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Chronic inflammation left unchecked can be quite harmful to the tissue with the pro-inflammatory stimulus. It is marked by the recruitment and activation of leukocytes, including lymphocytes and macrophages with their subsequent proliferation and reactive oxygen species release. Macrophages are also one of the primary players in propagating the inflammatory response as they secrete pro-inflammatory cytokines to sustain local tissue responses. Current therapies for chronic inflammation include non-steroidal anti-inflammatory drugs and glucocorticoids; however, both have various side effects and setbacks. Somatostatin is an endogenous hormone which inhibits cellular secretion and proliferation throughout the body. Somatostatin receptor activation is mediated through a family of heterotrimeric guanine nucleotide coupled proteins (G-proteins) belonging to the G_i and G_o family of G-proteins. In this study, we show that a murine macrophage cell line, RAW264.7, transcribes the mRNA and expresses the protein of the somatostatin receptor 2B subtype. We also demonstrate that this receptor reduces cytokine-induced phosphorylation of the STAT-3 transcription factor. Taken together, these data suggest the functional presence of a somatostatin receptor in the RAW 264.7 macrophage cell, a cellular model of the murine macrophage.

INTRODUCTION

Chronic inflammation is a prolonged, inflammatory response which persists after the initial actions of the acute inflammation response (Gilliland 1989; Geboes 1994). A hallmark of chronic inflammation is the involvement of monocytes and macrophages. Monocytes are blood-borne cells that, when stimulated, transmigrate through the endothelial layer of the blood vessel. The monocyte undergoes chemotaxis in response to soluble chemical mediators and, following diapedesis, differentiates into the tissue resident macrophage cell (Cross *et al.* 1997; Stout and Suttles 2004). Macrophages perform two key functions in inflammation, the generation of reactive oxygen species (ROS) production and the secretion of cytokine. ROS are rapidly released in response to a pro-inflammatory stimulus, but their damage extends to local tissues as well. Cytokines are the major chemical mediators that drive the chronic inflammatory process; the notable cytokines released by macrophages are TNF-α, IL-1 and IL-6 (Denis *et al.* 1991; Shacter *et al.* 1993). Macrophages and other immune cells in the inflamed tissue are activated by these pro-inflammatory cytokines, resulting in cellular proliferation and the secretion of additional cytokines (Badolato and Oppenheim 1996).

Chronic inflammatory diseases account for significant morbidity and mortality (Pleis and Lethbridge-Cejku 2006; Hootman *et al.* 2006). Examples include rheumatoid arthritis, inflammatory bowel diseases (e.g. Crohn's disease), tuberculosis, chronic

obstructive pulmonary disease and chronic cholecystitis. Many of the chronic inflammatory diseases are without cures; however, anti-inflammatory agents are used to help control the inflammatory process (Dale and Haylett 2004). Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used as many possess anti-inflammatory, analgesic and antipyretic actions. NSAIDs work to reduce vasodilatation, thereby, reducing leukocyte transmigration through the endothelial layer of the blood vessel. However, chronic NSAID use is associated with side-effects (Davies *et al.* 2006; Gooch *et al.* 2007). Gastrointestinal disturbances, skin reactions, adverse renal effects, bone marrow depression, liver disorders and even a type of encephalitis (Reye's syndrome) have been associated with large doses or long term use of NSAIDs. Other anti-inflammatory agents include glucocorticoids and anti-rheumatoid drugs (Dale and Haylett 2004).

The body's endogenous hormones are associated with either promoting or attenuating inflammatory responses. For instance the ovarian hormone, estrogen, is considered to be anti-inflammatory (Geraldes *et al.* 2006; Xing *et al.* 2007). Peptide hormones are also thought to be of considerable importance in maintaining an inflammatory balance. For example, corticotrophin-releasing hormone is thought to be pro-inflammatory (Zoumakis *et al.* 2000) while, the hormone somatostatin (Somatotropin inhibitory releasing hormone; SRIF) has been shown to be anti-inflammatory (Helyes *et al.* 2001). SRIF is an endogenous peptide shown to exert its effects on endocrine, neural, grastrointestinal and immune cells (Weckbecker 2003; Blake et al. 2004). Overall, SRIF controls cell proliferation and secretion. For example, SRIF modulates endothelial

inflammation and immune cell recruitment in coronary artery disease and has demonstrated clinical utility in treating refractory rheumatoid arthritis (Badway and Blake 2005; Blake et al., 2007). SRIF analogues have been used to control the hypersecretion of growth hormone in patients with acromegaly (Melmed *et al.* 2005). The anti-inflammatory properties of SRIF make it an attractive target for treatment of chronic inflammation.

SRIF exists as two active peptides in mammals, SRIF-14 and SRIF-28, both of which are derived from a common precursor, pre-prosomatostatin (Reichlin 1983). SRIF-14 and SRIF-28 are found in the gastrointestinal tract, central nervous system, immune cells and certain tumor cells, in which the peptides are produced in a tissue-selective manner (Patel 1999). SRIF-14 is mainly localized in the central nervous system, while SRIF-28 is located in the gastrointestinal and immune tissues (Reisine and Bell 1995; de Lecea 2005). SRIF-28 and SRIF-14 differ in the amino terminus with SRIF-28 possessing an amino-terminal extension of 14 amino acids compared to SRIF-14. SRIF-14 and SRIF-28 share a common pharmacophore amino acid sequence FWKT, which is held in a β-turn configuration through an internal cysteine disulfide linkage (Patel and Reichlin 1979). This pharmacophore sequence has facilitated the development of stable, peptidyl and non-peptidyl agonists and antagonists, (Weckbecker et al., 2003; Blake *et al.* 2004; Vaysee *et al.* 2005).

SRIF exerts its intracellular actions by binding to a family of homologous G-protein coupled receptors (GPCRs). The SRIF receptor family originates from five genes but includes six different receptors labeled sst_{1-5} with sst_2 having two splice variants, sst_{2A}

and sst_{2B}. The SRIF receptors are chiefly coupled to the G_i and G_o proteins which are known to exert predominately inhibitory actions in the cell (Hoyer *et al.* 1995). The SRIF receptor signaling cascade includes the control of intracellular cyclic nucleotide levels, reductions in protein phosphorylation states and the regulation of intracellular ion concentrations (Weckbecker 2003). The exact signaling cascades for these two events are dependent on receptor subtype and tissue distribution.

The RAW264.7 cell line was established from a tumor induced by Abelson murine leukemia virus in the BALB/c adult male mouse (Raschke *et al.* 1978). RAW 264.7 cells are extensively used in the study of mouse monocyte function as these cells possess a number of desirable features, including the ability to undergo transformation from monocytes to macrophages in response to a range of physiologically relevant stimuli. Two early studies have demonstrated that RAW 264.7 cells are responsive to SRIF peptides, yet the molecular target of this activity remains unknown (Bellocq et al. 1999; Ahmed et al. 2001). Recently, human macrophages have been shown to transcribe sst_1 and sst_2 mRNA, and a complex functional responsiveness has been shown (Armani *et al.* 2007).

In this study, we aim to identify the molecular target of the SRIF peptides in the RAW264.7 cell line. The goal of the present study was two-fold: (1) to determine if the sst_2 receptor splice variants sst2a and sst2b are expressed in RAW 264.7 cells and (2) demonstrate a function for the sst_2 receptor. The results of this study demonstrate that a subtype of the sst_2 receptor does functionally exist in the RAW264.7 cell line.

MATERIALS AND METHODS

Materials

The murine monocyte/macrophage cell line (RAW264.7) was purchased from American Type Culture Collection (Manassas, VA). Sterile cell culture plasticware was obtained from Mid-West Scientific (St Louis, MO). Cell culture media (RPMI and Opti-Mem) and washing buffers (Phosphate Buffered Saline) were purchased from Invitrogen Life Technologies (Carlsbad, CA). SRIF and LIF were purchased from Peninsula Labs (Belmont, CA) and Chemicon International (Temecula, CA). 18½ gauge syringe needles were obtained for Becton Dickinson and Company (Rutherford, NJ). cDNA was synthesized with use of the Superscript One-Step RT-PCR system from Invitrogen Life Technologies (Carlsbad, CA). Synthetic oligonucleotide primers were purchased from Invitrogen Life Technologies (Carlsbad, CA). Somatostatin receptor 2A and 2B antibodies, with the corresponding blocking peptides, were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA); the phospho-STAT-3 antibody was obtained from Cell Signaling Technology (Danvers, MA). SDS-PAGE and Western blotting supplies were purchased from Invitrogen Life Technologies (Carlsbad, CA). Chamber slides for confocal microscopy were obtained from Nunc Inc. (Naperville, IL). Donkey serum was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Vectashield sealant

and fluorescein-tagged secondary anitbodies were obtained from Vector Laboratories (Burlingame, CA).

Cell culture

The RAW264.7 cells were cultured in T-75 cm^2 flasks and 24 well cell culture dishes with Roswell Park Memorial Institute medium, RPMI, (supplemented with GlutaMAX™, pencillin (100U/mL)/streptomycin (100 mg/mL), and 10% fetal calf serum) at 37°C in a humidified atmosphere (5% CO_2/95% air). The cell cultures were passaged when the flasks reached ~75% confluence (by visual inspection) and allowed at least 48 hours to recover before experimentation.

Messenger RNA (mRNA) isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

Murine mRNA was isolated from the RAW264.7 cell line using the FastTrack® 2.0 mRNA Isolation Kit according to the manufacturer's instructions (Invitrogen Life Technologies, CA). RAW264.7 cells were washed in cold (4°C) phosphate buffered saline; the cells were then passed through an 18.5 gauge needle 4 times. Cell samples were lysed at 45°C for 20 minutes. The NaCl final concentration was then adjusted to 0.5M; subsequently, the lysates were passed through an 18½ gauge needle 4 times. Oligo dT cellulose was added to the lysates and incubated at room temperature for 60 minutes. Beads were washed and eluted according to manufacturer's instructions. The mRNA was

precipitated with 2M sodium acetate and 200 proof ethanol and frozen on dry ice. The mRNA was finally resuspended with the kit's elution buffer and stored at -80°C.

Isolated murine mRNA was reverse transcribed and amplified with the polymerase chain reaction using the Superscript One-Step RT-PCR system. cDNA synthesis was performed with an initial 50.0°C incubation for 30 minutes with reverse transcriptase; subsequent PCR [94.0°C, 15s; 60.0°C, 30s; 72.0°C, 30s] of 40 cycles and a final extension at 72.0°C for 5 minutes was sufficient to amplify the cDNA products. Primer sets described by Eliott *et al.* (1999) are: Total SSTR2-f = CTTGGCCATGCAGGTGGCGCTAGT, Total SSTR2-r = ATGGGGTTGGCGCAGCTGTTGG, SSTR2A-f = CTTGGCCATGCAGGTGGCGCTAGT, SSTR2A-r = TTGTCCTGCTTACTGTCGCTCCTCT, SSTR2B-f = CTTGGCCATGCAGGTGGCGCTAGT, SSTR2B-r = TCCGGATTGTGAATTGTCTGCCTTGA . A final reaction mixture (50 μL) consisted of 1X Reaction Mix Buffer containing SuperScript RT/Platinum Taq Mix, 10 μM forward and reverse primers and template mRNA. Thermal cycling was performed using an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA).

RT-PCR products were analyzed following horizontal gel electrophoresis with a 1.5% agarose gel in 1X TAE buffer (Invitrogen Life Technologies, CA) and stained with ethidium bromide (10 μg/mL). Digital images of the gel were obtained with aid of the Gel-Doc It 300 Imaging System and its corresponding software (UVP, Inc., CA).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

RAW264.7 cells were grown in 24-well plates with complete RPMI medium for 48 hours and starved on serum-free medium. The starved-cultured cells were incubated with and without 10 ng/μL LIF and 100 nM SRIF for 15 minutes at 37°C. After treatment, RAW264.7 cells were lysed with 1X NuPAGE LDS sample buffer. Cell lysates were passed through a 26½ gauge needle and heated at 70°C for 10 minutes, separated with NuPAGE 10% BIS-TRIS polyacrylamide gels , electroblotted onto polyvinyldifluoride (PVDF) membranes and treated for 1.5 hour in 10% (w/v) bovine serum albumin blocking buffer (Fluka Biochemicals/Sigma-Aldrich, St. Louis, MO). SSTR2A and SSTR2B were detected with a 1:200 dilution of SSTR2A or SSTR2B primary antibodies, respectively. Phospho-STAT3 was detected with a 1:2000 dilution of an affinity purified phospho-STAT3 antibody. Antibody binding to the PVDF membranes was detected using a horseradish peroxidase-conjugated secondary antibody at a 1:2000 dilution. The resulting immunoreactivity was detected using ECL chemiluminescence (GE Healthcare Life Sciences, Anaheim, CA). Digital images of the membrane were obtained with a STORM PhosphorImager (GE Healthcare, NJ), and densitometric analysis was performed with NIH ImageJ and quantified using GraphPad Prism 4.0 (GraphPad Software, Carlsbad, CA).

Fluorescence laser scanning confocal microscopy

RAW264.7 cells were grown in Lab-Tek® 4-well, 1.8 cm^2/well glass chamber slide with complete RPMI medium for 48 hours and starved in serum-media for 24 hours. The starved-cultured cells were incubated in the presence of absence of ligands for 15 minutes at 37°C. The cell monolayers were fixed with 4% paraformaldehyde, rinsed and incubated with a 1:100 of the SSTR2A or SSTR2B antibody in 1% normal donkey serum. A fluorescein-tagged secondary antibody, with 0.1μg/mL DAPI, was then incubated with the cell monolayers in 1% normal donkey serum. Peptide blockade of the antibodies was carried via an overnight incubation of the antibodies with a five-fold excess (by weight) of the blocking peptides at 4°C. Antibody stained cells were mounted with Vectashield. Scanning laser confocal microscopy (excitation wavelength = 495 nm, emission wavelength= 530 nm) was performed with an Olympus Fluoview™ FV1000 Confocal Microscope (Olympus, USA), and digital images were obtained with Olympus Fluoview FV10-ASW Version 1.3a software (Olympus, USA).

RESULTS

Somatostatin Receptor Subtype 2B mRNA present in RAW264.7 cells

The initial study on RAW 264.7 expression of SST receptor subtypes used isolated mRNA and RT-PCR, in conjunction with receptor subtype specific oligonucleotide primers. cDNA was transcribed from the isolated mRNA of the RAW264.7 cells and amplified using RT-PCR. Primers selective for the 2A and 2B subtypes (Fig. 1), used in conjunction with RT-PCR and agarose gel electrophoresis revealed a cDNA band at ~200 bp (Fig. 2). Specific primers for either the 2A or 2B subtype were used to further delineate which receptor subtype was present. Based upon these results (Fig. 2), the 2B receptor subtype appeared to be the dominant receptor subtype transcribed.

A band ~200 bp for cyclophilin B was observed as a positive control for RT-PCR. However, the negative control (null RT) contained the same size band indicating a possible genomic DNA contamination in the mRNA template. A null template control resulted in the same band as the negative RT-PCR control, suggesting the DNA contamination was present in the cylcophilin B primer stocks and not the isolated mRNA template.

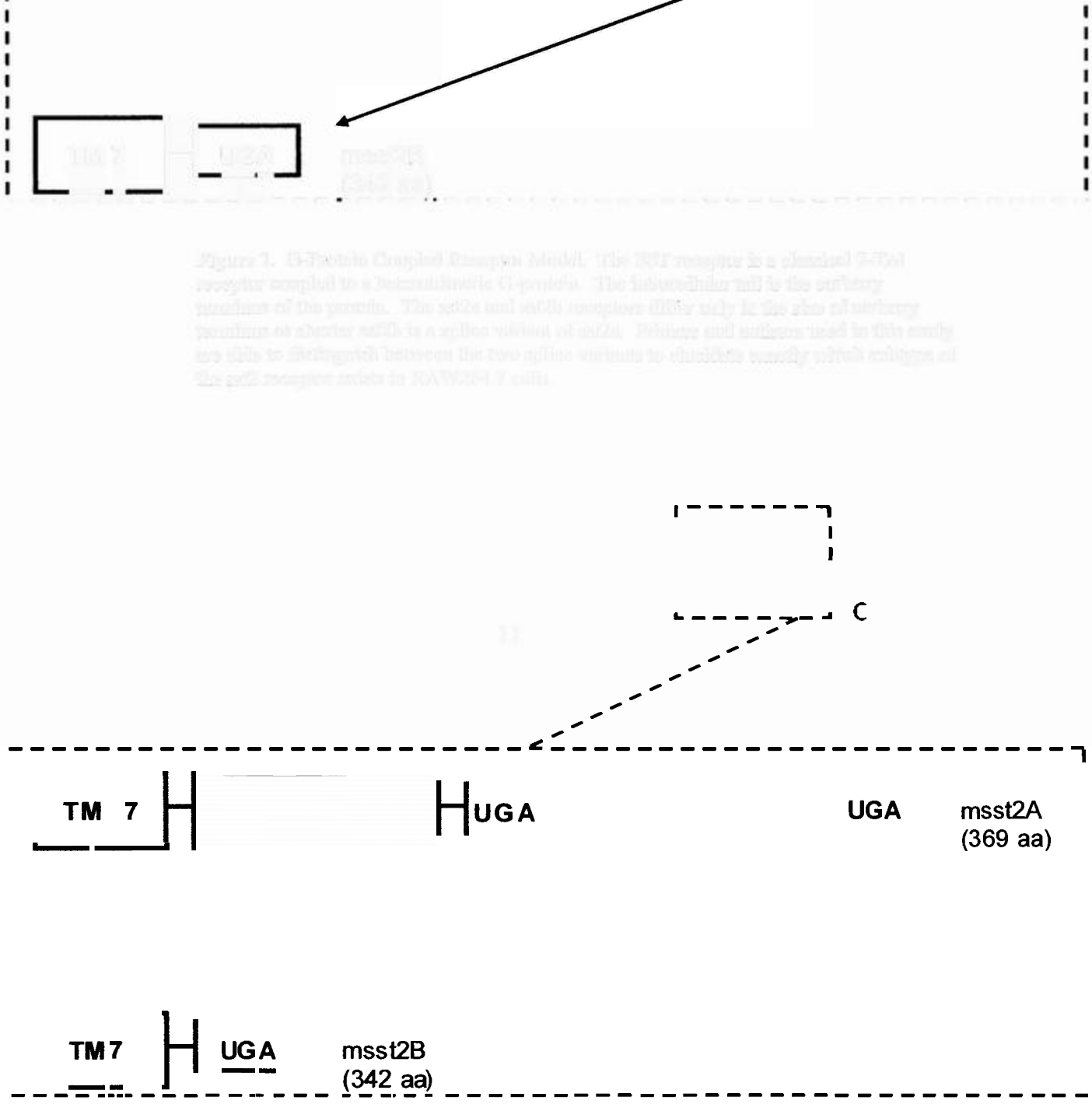


Figure 1. G-Protein Coupled Receptor Model. The SST receptor is a classical 7-TM receptor coupled to a heterotrimeric G-protein. The intracellular tail is the carboxy terminus of the protein. The sst2a and sst2b receptors differ only in the size of carboxy terminus as shorter sst2b is a splice variant of sst2a. Primers and antisera used in this study are able to distinguish between the two splice variants to elucidate exactly which subtype of the sst2 receptor exists in RAW264.7 cells.

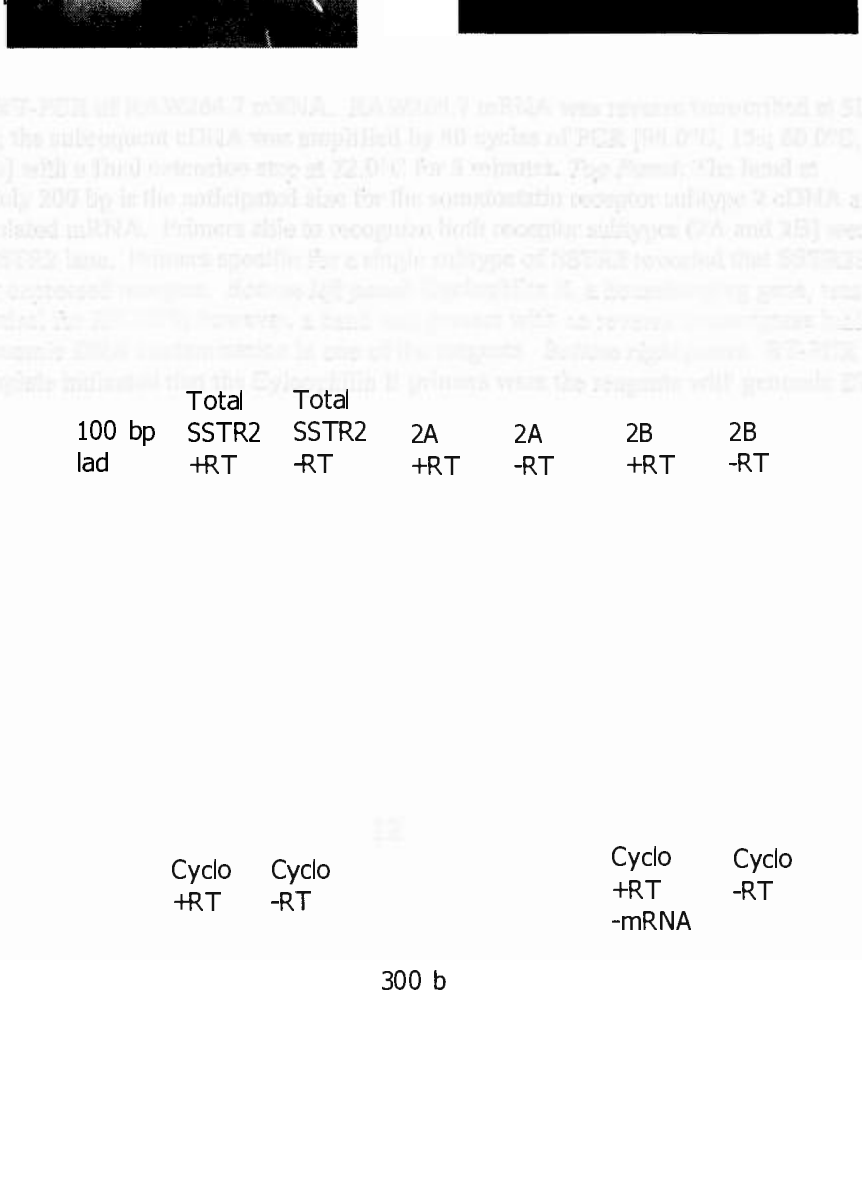


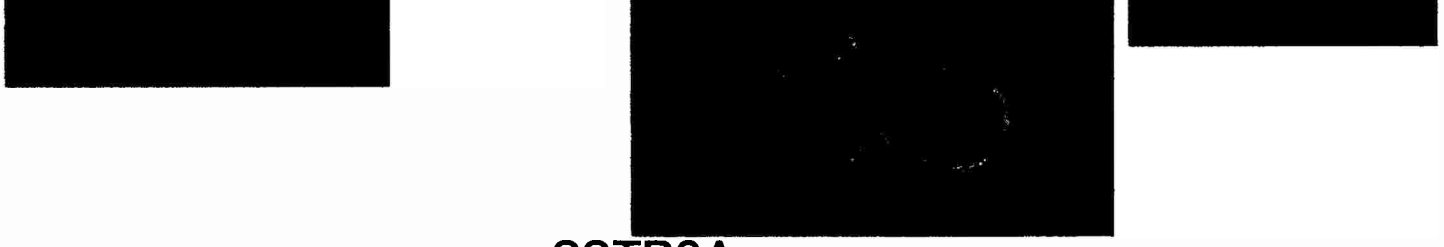
Figure 2. RT-PCR of RAW264.7 mRNA. RAW264.7 mRNA was reverse transcribed at 50.0°C for 30 minutes; the subsequent cDNA was amplified by 40 cycles of PCR [94.0°C, 15s; 60.0°C, 30s; 72.0°C, 30s] with a final extension step at 72.0°C for 5 minutes. *Top Panel*: The band at approximately 200 bp is the anticipated size for the somatostatin receptor subtype 2 cDNA amplified from the isolated mRNA. Primers able to recognize both receptor subtypes (2A and 2B) were used in the Total SSTR2 lane. Primers specific for a single subtype of SSTR2 revealed that SSTR2B is the dominantly expressed receptor. *Bottom left panel*: Cyclophilin B, a housekeeping gene, was used as a positive control for RT-PCR; however, a band was present with no reverse transcriptase indicating possible genomic DNA contamination in one of the reagents. *Bottom right panel*: RT-PCR with no mRNA template indicated that the Cylcophilin B primers were the reagents with genomic DNA.

Somatostatin Receptor 2B protein expressed in RAW264.7 cells

Antibodies for the sst2a and sst2b receptor subtypes were obtained from a commercial source. Each antibody recognizes one of the two splice variants of the sst2 receptor carboxyl terminal amino acid sequences (Patel *et al.* 1994). We used these two antibodies to determine which sst2 receptor splice variant might be expressed in the RAW 264.7 cells. Total cell lysates were prepared from RAW monolayer cultures, resolved by SDS-PAGE, electroblotted and probed with either the sst2a or sst2b selective antiserum. As shown in the top panels of Figure 3, the somatostatin receptor 2b receptor is endogenously expressed in RAW264.7 cells. AtT-20 cells, a murine adenocorticotroph cell line that expresses sst2a and sst2b, as well as sst5 (Sarret et al., 1998; Strowski et al., 2002), serves as a positive control for the sst2a and sst2b subtypes. We observed that the sst2a selective antiserum recognized an AtT-20 cell protein band at approximately 40 kDa, in agreement with earlier studies, but that the sst2a antiserum did not appear to recognize a corresponding band in the RAW 264.7 cell samples (Fig. 3). In contrast, the sst2b antiserum detected a protein band at ~40 kDa, which was present in both the RAW 264.7 cell lysates and in the AtT-20 cell control lysates (Fig. 3). Based upon the results obtained from the RT-PCR experiments and the Western blotting experiments, it appears that only the sst2b receptor is present in RAW 264.7 cells.

Indirect immunofluorescence, in conjunction with laser scanning confocal microscopy was used to further establish the presence of the sst2b receptor in individual RAW 264.7 cells (Fig. 4). Nuclear DNA staining was visualized with 4',6-diamidino-2-phenylindole (DAPI) in conjunction with the sst2a and sst2b polyclonal antisera. Indirect

Figure 3. Western Blot analysis using the somatostatin receptor subtype selective antibodies. Antibodies against the two somatostatin receptor 2 subtypes were used to identify which proteins were present in RAW264.7 (R) cells. Western blot scans using either the 2A (top panel) or 2B (bottom panel) selective antibodies as probes are shown. AtT-20 (A) cells are positive controls for both subtypes. Evidence for the 2B receptor in the RAW264.7 cells is seen with the band at ~40 kDa.



SSTR2A

SSTR2B

Figure 4. Confocal Microscopy using the subtype selective antibodies. Antibodies against the two somatostatin receptor 2 subtypes were used to identify which proteins were present in paraformaldehyde-fixed RAW264.7 cells. *Top panels (A-D)*: RAW264.7 cells incubated with the 2A antibody. Control treatments lacking the primary antibody (Panel A) and the secondary antibody (Panel B) are shown. Primary and secondary antibody treatment reveals diffuse cytoplasmic staining of the 2A antibody in RAW 264.7 cells (Panel C). Peptide blockade of the 2A antibody serves as a negative control (Panel D). *Bottom panels (E-H)*: RAW264.7 cells incubated with the 2B antibody. Control treatments lacking the primary antibody (Panel E) and the secondary antibody (Panel F) are shown. Primary and secondary treatment reveals organelle-membrane localized 2B antibody binding in the RAW264.7 cells (Panel G). Peptide blockade of the 2B antibody serves as a negative control (Panel H). Nuclear staining was obtained with DAPI.

immunofluorescence staining with the sst2a antisera demonstrated a diffuse cytoplasmic pattern, suggesting significant non-specific binding (Fig. 4, panel C). In contrast, the sst2b antibody staining pattern exposes an intense signal in a highly localized fashion suggesting presence of this receptor subtype in the RAW264.7 cells (Fig. 4, panel G). Incubation of the sst2a and 2b antibodies with their respective blocking peptides before antibody treatment to the cells resulted in an absence of fluorescence when the treated cells were imaged (Fig. 4, panels D and H). Peptide blockade of antibodies serves as a control for antibody specificity. These peptide blockade results suggest that the antibodies are binding to the somatostatin receptor proteins.

Taken together, the molecular data from the RT-PCR, Western Blot and Confocal Microscopy experiments show evidence for the endogenous expression of the somatostatin receptor 2B subtype protein in RAW264.7 cells.

Somatostatin Receptor 2 subtypes reduce cytokine-induced STAT-3 phosphorylation

With evidence for sst2b receptor expression in RAW264.7 cells, we next sought functional evidence for the sst2b receptor protein. To show sst2b receptor function, RAW264.7 cells were treated with10 ng/μL LIF (pro-inflammatory cytokine) and/or 100 nM SRIF-14. Treated cells were lysed, resolved, electroblotted and probed with a phospho-specific STAT-3 antibody (Fig. 5). Densitometric analysis of the resulting Western Blot scans revealed a significant ($p<.01$) cytokine-induced increase in STAT-3 phosphorylation. SRIF-14 treatment alone did not significantly vary from basal phoshorylation levels; however, SRIF-14 co-treatment with LIF resulted in a significant

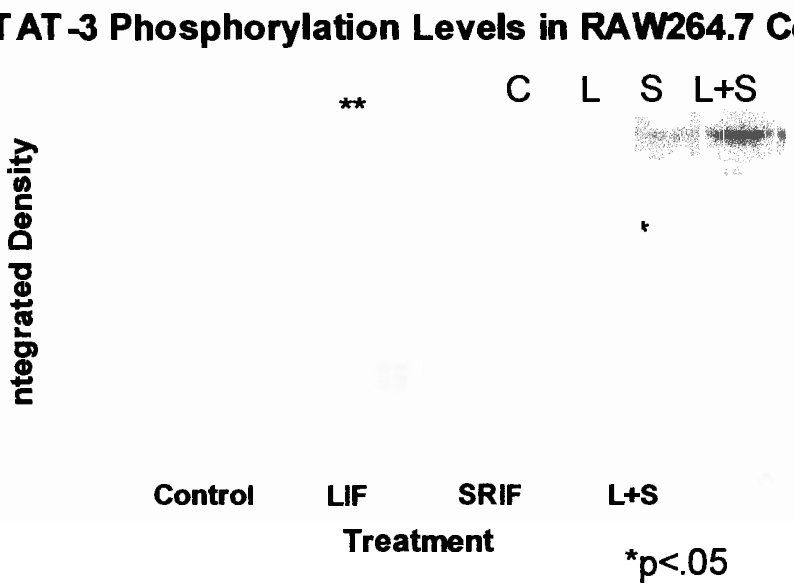


Figure 5. SSTR activation decreases cytokine-induced STAT-3 phosphorylation, as determined with a STAT-3 phospho-specific antiserum. RAW264.7 cells were treated with 100 ng/μL LIF and/or 100 nM SRIF-14 for 15 minutes at 37°C. *Top right panel*: A representative Western blot scan using a phospho-specific STAT-3 antibody. *Bar graph*: Densitometric analysis of the Western blot scans shows densitometric data against control. LIF significantly increases STAT-3 phosphorylation ($p<.01$); however, SRIF co-treatment resulted in a significant decrease in STAT-3 phosphorylation ($p<.05$). All data are significant at $p < .05$.

($p<.05$) reduction in STAT-3 phosphorylation levels when compared to the LIF treatment. This result shows functional evidence for the sst2b receptor in RAW264.7 cells.

Taken together, the data from the RT-PCR, Western blot, confocal microscopy experiments and functional assays describe the functional expression of the somatostatin 2B receptor in RAW264.7 cells.

DISCUSSION

Chronic inflammation serves as a vital tool employed by the body to deal with harmful stimuli that are not easily rid of through acute inflammation. Left unchecked, however, chronic inflammation can progress from a nuisance into a life-threatening disease. The delicate balances of the chemical mediators that control chronic inflammation are paramount to the body's ability to recover from this less-than-desirable condition. Up-regulation in the production of these chemical mediators (e.g. cytokines) sustains the chronic inflammatory response in the origin of the stimulus. Macrophage involvement is a hallmark of chronic inflammation; macrophages facilitate two very destructive processes in the eyes of the host tissue: reactive oxygen species production and cytokine secretion. Reactive oxygen species can significantly damage host tissue, whereas cytokines are the chemical mediators that encourage macrophage proliferation and secretion of more pro-inflammatory cytokines. Over time these processes will concur to subject host tissues to devastating effects, which is why effective treatments for chronic inflammatory diseases are absolutely necessary.

In this study we show that a murine macrophage cell line (RAW264.7) functionally expresses the somatostatin receptor subtype 2B. Reverse transcription of isolated mRNA from these cells reveals that, indeed, these cells do express the transcript for the somatostatin receptor 2 subtype. Primers specific for the subtypes of the 2

receptor delineate that the 2B receptor is the major subtype that is transcribed in these cells. Cyclophilin B served as a positive control for this experiment as it is a housekeeping gene which is consistently expressed in peripheral blood cells (Pachot *et al.* 2004). One disturbing result is the presence of a band in the no RT control of the cyclophilin B RT-PCR. Subsequent control experiments (i.e. no template controls) resulted in the same band in the no RT treatment; this concludes then that the cyclophilin B primers, not the mRNA template, were contaminated with genomic DNA.

Once we identified that these cells transcribe the correct mRNA, we sought to find the expressed protein using immunolabeling. Western blot and confocal microscopy showed that the RAW264.7 cells did indeed express the 2B subtype of the somatostatin receptor. The non-specific, cytoplasmic binding pattern of the 2A receptor antibody suggested that the protein was not highly expressed at basal conditions; on the other hand, the 2B receptor antibody binding pattern showed a high degree of localization around the nucleus. We have not identified what compartment of the cell the binding is localized in (i.e. endoplasmic reticulum versus plasma membrane), but this binding pattern gives us significant evidence that indeed the 2B receptor subtype is expressed at basal conditions in these murine macrophage cells. Successful peptide blockade of the antibodies suggest that the antibodies are specific enough for our uses.

Functionality of the identified receptor was the next thing to establish in this study. Leukemia inhibitory factor (LIF)-, a member of the IL-6 cytokine family, induced phosphorylation of the transcription factor STAT-3 was readily observed as expected. Co-treatment of LIF with an agonist for the somatostatin receptor 2B subtype (SRIF-14)

significantly decreased the phosphorylation of STAT-3. This result suggests that there is a possible crosstalk between the SRIF GPCR and the IL-6 family of receptor tyrosine kinases. Indeed, somatostatin decreases IL-6 secretion by LPS-activated human monocytes (Peluso *et al.* 1996). A proposal for the junction between these two pathways is at phosphotyrosine phosphatases (PTPs). Somatostatin receptors are known to activate PTPs which lead to downstream events such as increased apoptosis and decreased cell proliferation (Reyl and Lewin 1982). In this model, an activated PTP by the somatostatin receptor 2B subtype dephosphorylates the Janus kinase-phosphorlyated STAT-3 transcription factor. STAT-3 translocation into the nucleus is dependent upon phosphorylation and dimerization and it promotes the transcription of proliferative and pro-inflammatory genes. PTP activation could inhibit this dimerization event and result in decreased cell proliferation and reduced cytokine production and secretion.

Somatostatin is an endogenous peptide which exerts many inhibitory effects in the body. Its anti-prolfierative and –secretive functions seen in various tissues of the body suggest it may be a favorable target for chronic inflammatory diseases (Weckbecker 2003). This study describes the functional presence of the somatostatin 2B receptor in a murine macrophage cell line. These results give hope to the idea that somatostatin receptor activation, via small-molecule mimics of the somatostatin peptide, will decrease the proliferation and cytokine secretion of chronically activated macrophages and serve therapeutic applications for chronic inflammatory diseases.

LITERATURE CITED

Ahmed, A., Wahbi, A., and Nordlin, K. Neuropeptides modulate a murine monocyte/macrophage cell line capacity for phagocytosis and killing of Leishmania major parasites. Immunopharmacology and Immunotoxicology. 23(3): 397-409(2001).

Armani, C., Catalani, E., Balbarini, A., Bagnoli, P., and Cervia, D. Expression, pharmacology, and functional role of somatostatin receptor subtypes 1 and 2 in human macrophages. Journal of Leukocyte Biology. 81: 845-855 (2007).

Auernhammer, C.J., Kopp, F.B., Vlotides, G., Dorn, F., Isele, N.B., Spottl, G., Cengic, N., Weber, M.M., Senaldi, G., and Engelhardt, D. Comparative Study of gp130 Cytokine Effects on Corticotroph AtT-20 Cells – Redundancy or Specificity of Neuroimmunoendocrine Modulators? Neuroimmunomodulation. 11: 224-232 (2004).

Badolato, R. and Oppenheim, J. Role of cytokines, acute-phase proteins, and chemokines in the progression of rheumatoid arthritis. Seminars in Arthritis and Rheumatism. 26(2): 526-538 (1996).

Badway, A.C., and Blake, A.D. Somatostatin: A Hormone for the Heart? Current Vascular Pharmacology. 3: 125-131 (2005).

Bellocq, A., Doublier, S., Suberville, S., Perez, J., Escoubet, B., Fouqueray, B., Puyol, D., and Baud, L. Somatostatin increases glucocorticoid binding and signaling in macrophages by blocking the calpain-specific cleavage of Hsp 90. Journal of Biological Chemistry. 274 (52): 36891-6 (1999).

Blake, A., Badway, A. and Strowski, M. Delineating neuronal somatostatin receptor signaling. Current Drug Targets: CNS and Neurological Disorders. 3:153-160 (2004).

Blake, A., West, F.M., Ghafoor, S., and Efthimiou, P. Somatostatin involvement in rheumatoid arthritis. Current Rheumatology Reviews. 3: 31-36 (2007).

Chowers, Y., Cahalon, L., Lahav., M., Schor, H., Tal, R., Bar-Meir, S., and Levite, M. Somatostatin through its specific receptor inhibits spontaneous and TNF-α- and bacteria-induced IL-8 and IL-1β secretion from intestinal epithelial cells. The Journal of Immunology. 165: 2955-2961 (2000).

Conchello, J.A., and Lichtman, J.W. Optical sectioning microscopy. Nature Methods. 2: 920-931 (2005).

Cross, A., Richardson, V., Ali, S., Palmer, I., Taub, D., and Rees, R. Migration responses of human monocytic cell lines to alpha- and beta-chemokines. Cytokine. 9(7): 521-528 (1997).

Dale, M., and Haylett. D. Pharmacology Condensed. Churchill Livingstone, 2004.

Davies, N., Reynolds, J., Undeberg, M., Gates, B., Ohgami, Y. and Vega-Villa, K. Minimizing risks of NSAIDs: cardiovascular, gastrointestinal and renal. Expert Review of Neurotherapeutics. 6(11): 1643-1655 (2006).

de Lecea, L. Cortistatin: a natural somatostatin analog. Journal of Endocrinological Investigation. 28 (11 Suppl): 10-14 (2005).

Denis, M., Cormier, Y., Tardif, J., Ghadirian, E. and Laviolette, M. Hypersensitivity pneumonitis: whole Micropolyspora faeni or antigens thereof stimulate the release of proinflammatory cytokines from macrophages. American Journal of Respiratory Cell and Molecular Biology. 5(2): 198-203 (1991).

Geboes, K. From inflammation to lesion. Acta Gastro-Enterologica Belgica. 57(5-6): 273-284 (1994).

Geraldes, P., Gagnon, S., Hadjadj, S., Merhi, Y., Sirois, M., Cloutier, I. and Tanguay, J. Estradiol blocks the induction of CD40 and CD40L expression on endothelial cells and prevents neutrophil adhesion: an ERalpha-mediated pathway. Cardiovascular Research. 71(3): 566-573 (2006).

Gilliland, B. Rheumatoid arthritis: a model of chronic inflammation. Arzneimittelforschung. 39(8A): 952-956 (1989).

Gonzalez-Rey, E., Chorny, A., Robledo, G., and Delgado, M. Cortistatin, a new anti-inflammatory peptide with therapeutic effect on lethal endotoxemia. The Journal of Experimental Medicine. 3: 563-571 (2006).

Gooch, K., Culleton, B., Manns, B., Zhang, J., Alfonso, H., Tonelli, M., Frank, C., Klarenbach, S. and Hemmelgarn, B. NSAID use and progression of chronic kidney disease. American Journal of Medicine. 120(3): e1-7 (2007).

Gordon., S., and Taylor, P.R. Monocyte and macrophage heterogeneity. Nature Reviews Immunology. 5: 953-963 (2005).

Hannon, J.P., Petrucci, C., Fehlmann, D., Viollet, C., Epelbaum, J., and Hoyer, D. Somatostatin sst_2 receptor knock-out mice: localization of sst_{1-5} receptor mRNA and binding in mouse brain by semi-quantitative RT-PCR, in situ hybridization histochemistry and receptor autoradiography. Neuropharmacology. 42: 396-413 (2002).

Helyes, Z., Pinter, E., Nemeth, J., Keri, G., Than, M., Oroszi, G., Horvath, A. and Szolcsanyi J. Anti-inflammatory effect of synthetic somatostatin analogues in the rat. British Journal of Pharmacology. 134(7): 1571-1579 (2001).

Hootman,. J., Bolen, J., Helmick, C. and Langmaid G. "Prevalence of Doctor-Diagnosed Arthritis and Arthritis-Attributable Activity Limitation --- United States, 2003—2005." MMWR. 2006. Center for Disease Control. 22 May 2007 <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5540a2.htm>

Hoyer, D., Bell, G., Berelowitz, M., Epelbaum, J., Feniuk, W., Humphrey, P., O'Carroll, A-M., Patel Y., Schronbrunn, A., Taylor, J. and Reisine, T. Classification and nomenclature of somatostatin receptors. Neuroscience Trends. 16: 86-90 (1995).

Kumar, U., Sasi, R., Suresh, S., Patel, A., Thangaraju, M., Metrakos, P., Patel, S.C., and Patel, Y.C. Subtype-selective expression of the five somatostatin receptors (hSSTR1-5) in human pancreatic islet cells: a quantitative double-label immuno-histochemical analysis. Diabetes. 48: 77-85 (1999).

Melmed, S., Casaneuva, F., Cavagnini, F., Chanson, P., Frohmna, L., Gaillard, R., Ghigo, E., Ho, K., Jaquet, P., Kleinberg, D. Lamberts, S., Laws, E., Lombardi, G., Sheppard, M., Thorner, M., Vance, M., Wass, J., and Giustina, A. Consensus statement: medical management of acrogmegaly. European Journal of Endocrinology. 6: 737-740 (2005).

Mocsai, A., Abram, C.L., Jakus, Z., Hu, Y., Lanier, L.L., and Lowell, C.A. Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. Nature Immunology. 7: 1326-1333 (2006).

Nguyen, V.T., and Gao, B. Cross-talk between α_{1B}-Adrenergic Receptor (α_{1B}AR) and Interleukin-6 (IL-6) Signaling Pathways. The Journal of Biological Chemistry. 274: 35492-35498 (1999).

Patel, Y.C., Panetta, R., Escher, E., Greenwood, M., and Srikant, C.B. Expression of Multiple Somatostatin Receptor Genes in AtT-20 Cells. The Journal of Biological Chemistry. 269: 1506-1509 (1994).

Patel, Y.C. Somatostatin and its receptor family. Frontiers in Neuroendocrinology. 20: 157-198 (1999).

Patel, Y. and Reichlin, S. Somatostatin. Methods of Hormone Radioimmunoassay. 2: 77-99 (1979).

Peluso, G., Petillo, O., Melone, M., Mazzarella, G., Ranieri, M., and Tajana G. Modulation of cytokine production in activated human monocytes by somatostatin. Neuropeptides. 30 (5): 443-51 (1996).

Perez, J., Viollet, C., Doublier, S., Videau, C., Epelbaum, J., and Baud, L. Somatostatin binds to murine macrophages through two distinct subsets of receptors. Journal of Neuroimmunology. 138: 38-44 (2003).

Pleis, J. and Lethbridge-Cejku M. "Summary Health Statistics for U.S. Adults: National Health Interview Survey, 2005." Vital and Health Statistics. Center for Disease Control: National Center for Health Stastistics. 10(232): 1-163 (2006).

Raschke, W., Baird S., Ralph, P. and Nakoinz, I. Functional macrophage cell lines transformed by Abelson leukemia virus. Cell. 15(1): 261-267 (1978).

Reardon, D.B., Dent, P., Wood, S.L., Kong, T., and Sturgill, T.W. Activation of *in vitro* of somatostatin receptor subtypes 2, 3, or 4 stimulates protein tyrosine phosphatase activity in membranes from transfection Ras-transformed NIH 3T3 cells: coexpression with catalytically inactive SHP-2 blocks responsiveness. Molecular Endocrinology. 11: 1062-1069 (1997).

Reichlin, S. Somatostatin. New England Journal of Medicine. 309: 1556-1563 (1983).

Reyl, F., and Lewin, M. Intracellular receptor for somatostatin in gastric mucosal cells: decomposition and reconstitution of somatostatin-stimulated phosphoprotein phosphatases. Proc. Natl Acad. Sci. USA. 79: 978–982 (1982).

Rocheville, M., Lange, D.C., Kumar, U., Patel, S.C., Patek, R.C., and Patel, Y.C. Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity. Science. 288: 154-157 (2000).

Reisine, T. and Bell G. Molecular biology of somatostatin receptors. Endocrine Reviews. 16: 427-442 (1995).

Rocheville, M., Lange, D.C., Kumar, U., Sasi, R., Patel, R.C., and Patel, Y.C. Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. The Journal of Biological Chemistry. 275: 7862-7869 (2000).

Sarret, P., Botto, J.M., Vincent, J.P., Mazella, J., and Beaudet, A. Preferential expression of sst2A over sst2B somatostatin receptor splice variant in rat brain and pituitary. Neuroendocrinology. 68: 37-43 (1998).

Shacter, E., Arzadon, G., and Williams, J. Stimulation of interleukin-6 and prostaglandin E2 secretion from peritoneal macrophages by polymers of albumin. Blood. 82(9): 2853-2864 (1993).

Sharma, K., Patel, Y.C., and Srikant, C.B. C-terminal region of human somatostatin receptor 5 is required for induction of Rb and G1 cell cycle arrest. Molecular Endocrinology. 13: 82-90 (1999).

Stout, R. and Suttles, J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. Journal of Leukocyte Biology. 76(3): 509-513 (2004).

Strowski, M.Z., Dashkevicz, M.P., Parmar, R. M., Wilkinson, H.A., Kohler, M., Schaeffer, J.M. and Blake, A.D. Somatostatin receptor subtypes sst_2 and sst_5 regulate corticotropin-releasing hormone stimulated adrenocorticotropin secretion from AtT-20 cells. Neuroendocrinology 75: 339-346 (2002).

ten Boku, A., Hofland, L. and van Hagen, P. Somatostatin and somatostatin receptors in the immune system: a review. European Cytokine Network. 11: 161-176 (2000).

Vaysee, N., Lahlou, H., Ferjoux, G. and Susini, C. Novel therapeutic targets for somatostatin in chronic inflammatory disease. Current Medicinal Chemistry-Anti-inflammatory and Anti-allergy Agents. 4:91-104 (2005).

Weckbecker, G., Lewis, I., Albert, R., Schmid, H.A., Hoyer, D., and Bruns, C. Opportunities in somatostatin research: biological, chemical and therapeutic aspects. Nature Reviews Drug Discovery. 2: 999-1017 (2003).

Xing, D., Feng, W., Miller, A., Weathington, N., Chen, Y., Novak, L., Blalock, J. and Oparil S. Estrogen Modulates TNF-{alpha}-Induced Inflammatory Responses in Rat Aortic Smooth Muscle Cells through Estrogen Receptor-{beta} Activation. American Journal of Physiology: Heart and Circulatory Physiology. E-published ahead of print (2007).

Zoumakis, E., Margioris, A., Stournaras, C., Dermitzaki, E., Angelakis, E., Makrigiannakis, A., Koumantakis, E. and Gravanis, A. Corticotrophin-releasing hormone (CRH) interacts with inflammatory prostaglandins and interleukins and affects the decidualization of human endometrial stroma. Molecular Human Reproduction. 6(4): 344-351 (2000).