Cytokine and neurotransmitter regulation of glial cell production of IL-6; indications for neurodegenerative disorders

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ABSTRACT

Cytokine and Neurotransmitter Regulation of Glial Cell Production of IL-6; Indications for Neurodegenerative Disorders

by

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There are two basic types of cells in the human brain: glial cells and neurons. Neurons are responsible for the relay of information within the brain and between the brain and other organs. Glial cells represent two cell groups microglia (macrophages) and macroglia (astrocytes and oligodendrocytes). Activated microglia are the main generators of IL-1. Activated immunoreactive IL-1 microglia are increased sixfold in the central nervous system (CNS) of Alzheimer disease (AD) patients compared to age-matched controls and are associated with neuritic plaques. There is increasing evidence that plaque-associated activated glia and cytokines are important factors in the development and progression of the neuropathological hallmarks of AD.

Cytokines are mediators of the immune response and have the ability to affect neuroendocrine function and hormone secretion. The cytokines interleukin-1 (IL-1) and interleukin-6 (IL-6) are produced during an immune reaction and cause inflammation and swelling of the surrounding tissue. AD is a neurodegenerative disorder characterized by
well-defined neuropathologic entities. Both IL-1 and IL-6 are present in senile plaques and may cause a self-propagating inflammatory process that contributes to the disease processes of AD.

Somatostatin (SRIF) is a small, inhibitory neuropeptide present throughout the CNS. There are two types of SRIF found in mammals, SRIF-14 and 28. SRIF contributes to the maintenance of neuronal health in the adult and aging brain. AD causes the degeneration of somatostatin-containing neurons and an overall decrease of SRIF in the CNS. This decrease of SRIF correlates with the distribution of senile plaques, neurofibrillary tangles and the cognitive degeneration of the patient. Because SRIF is an inhibitory neuropeptide we sought to determine the effects of SRIF on the production of IL-6 in rat C6 glial tumor cells. The release of IL-6 in the C6 cell conditioned media was quantified using the IL-6 dependent 7TD1 hybridoma bioassay.

Both SRIF-14 and 28 significantly reduced the IL-1 stimulation of IL-6 released from C6 glioma cells in vitro. Isoproterenol (IPT) is a drug that mimics the actions of the neurotransmitter norepinephrine. IPT in the presence of IL-1 causes a synergistic stimulation of IL-6 release from the C6 glioma cells. In addition, SRIF-14 and SRIF-28 inhibited the IL-1 plus IPT synergistic induction of IL-6 release.

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter located in the CNS. It is one of several amino acids that play a part in regulating the nervous system. GABA is formed from glutamic acid, one of the traditional amino acids. When activated the GABA_A receptor causes an increase in the net influx of Cl^- ions, which causes hyperpolarization of the neuron. Hyperpolarization of the neuron inhibits the action of excitatory neurotransmitters by raising the threshold for the onset of action potentials.

We decided to investigate GABA ability to inhibit the release of IL-6 by IL-1β.
GABA inhibited the basal and IL-1β stimulated release of IL-6. Further GABA is shown to inhibit the synergistic release of IL-6 by IL-1β and the β-adrenergic receptor agonist IPT.

Previously shown LPC induces the release of IL-6 from the C6 glioma cell line. This induction of IL-6 suggests that LPC acts as a second messenger of IL-1β in the C6 cell line. As with IL-1β, LPC-18 and IPT together will produce a synergistic release of IL-6. This increased release can be inhibited by the addition of SRIF-14.

To better understand the signal pathways of IL-1β that results in the release of IL-6 we used the inhibitors SP600125 and SB203580. SB203580 is a p38 MAPK inhibitor that targets the p38α and p38β2 kinases, while the p38β kinase exhibits only partially sensitivity. The inhibitor SP600125 targets the JNK kinase.

The SB203580 kinase inhibitor resulted in the reduction of IL-6 below detectable levels both basally and with the addition of IL-1β or LPC. SP600125 on the other hand showed no inhibition of the release of IL-6 for either IL-1β or LPC.

It may be possible to reduce the high levels of the cytokine IL-6 by increasing the amount of SRIF in the CNS back towards normal levels. We suggest that the ability to lower the concentration of IL-6 maybe therapeutic for AD patients. Providing a new method of treatment for AD.
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CHAPTER 1

INTRODUCTION

Hypothesis Statement

The release of IL-6 is an important facet of the immune system and regulation of the neuroendocrine system. Its release has ramifications on a number of factors in the CNS. When its release is not properly regulated as in AD and other neurodegenerative disorders it can cause damage to surrounding tissue and further the progression of the disease. Previous studies show that the release of IL-6 from the C6 cell line can be increased by a number of neurotransmitters such as norepinephrine and epinephrine. In this study, I am investigating the neurotransmitters SRIF-14, SRIF-28, GABA and the SP600125 and SB203580 inhibitors. I hypothesize that the neurotransmitters SRIF-14, SRIF-28 and GABA will reduce the release of IL-6 from the C6 cell line. Further I hypothesize that the SB203580 p38 MAPK kinase inhibitor will reduce the release of IL-6, while the SP600125 JNK kinase inhibitor will not affect the release of IL-6 from the C6 cell line. The findings from this study will further elucidate the IL-1β signal transduction pathway for the stimulation of IL-6 from the rat C6 glioma cell line. More importantly the ability to regulate IL-6 in the CNS of AD patients may prove to have therapeutic value in slowing down the progression of this disease.
Immune System

There are two main components of the immune system, the humoral immune response and the cell-mediated immune response. The humoral or antibody response involves the concerted action of two different classes of white cells or lymphocytes, which are the B cells and T cells. When B cells are exposed to foreign substances or antigens they multiply and begin producing immunoglobulin proteins (antibodies), which are then secreted into the bloodstream and bind to the target antigen. The T cells have three different classes: killer T cells that destroy target cells, helper T cells that stimulate the maturation of antigen-stimulated B and T cells and suppressor T cells that block the response of the helper T cells. The T cells also regulate the inflammatory response by the secretion of specific interleukin proteins.

The cell-mediated immune response has two forms of action that involve the use of different types of T cells. The specific death T cells, T_D, can secrete interleukins that activate macrophages after interaction with an antigen. This interaction produces a slow inflammatory response. The second form of action involves the killer T cell, T_K, which will cause the lysis of target cells with antigen bound to them.

Neuroendocrine System

The neuroendocrine system is made up of specialized neuroendocrine cells found in the hypothalamic region of the brain that can regulate the secretion of hormones from the pituitary gland (Bowman, 1999). The hypothalamic-median eminence-anterior pituitary neuropeptide system and the hypothalamic-posterior pituitary system form the pathways by which the brain and neuroendocrine secretions reach their targets. The hypothalamus is essential in the maintenance of homeostasis and integration of behavioral patterns. It
has reciprocal neural connections with much of the cerebral hemispheres and brainstem. In addition many of the hypothalamic areas contain neurosecretory cells that can regulate the release of pituitary tropic hormones through a vascular route to the anterior pituitary gland, or through a neural route to the posterior pituitary (Strand, 1999).

There are two main components to this system. The first component is the magnocellular neuroendocrine system made up of large cells in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus. The axons from these cells pass through the infundibulum to terminate in the posterior pituitary gland, where they release their secretions, oxytocin (OT) and vasopressin (VT), into the venous circulation (Strand, 1999).

The parvocellular neurosecretory system forms the second component. The axons of cells in several parts of the hypothalamic region, including the preoptic area, ventromedial, dorsomedial, posterior hypothalamic, premammillary and suprachiasmatic nuclei release their stimulating or inhibiting secretions into the vascular system of the median eminence (ME).

Interleukin Cytokines

Cytokines are low molecular weight proteins secreted by a cell into the extracellular fluid where they exert their effects on the same cells (autocrine activity) or on neighboring cells (paracrine activity) by interacting with specific receptors (Balkwill, 1991). Through these actions cytokines can mediate immune responses as well as affect neuroendocrine function and hormone secretion. The cytokine family of peptides consists of the interleukins (1 to 18), tumor necrosis factor-α (TNF-α) and -β, colony stimulating factor (CSF), interferons, transforming-growth factors-β, chemokines and
growth factors (Casey, 1999). As a subset of the cytokine family, the interleukins function as inflammatory cytokines by promoting cell growth, differentiation and functional activation. IL-4 is the exception that functions as an anti-inflammatory cytokine. These inflammatory cytokines include interleukin-1 (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6) and tumor necrosis factor-α. They are made chiefly by activated macrophages. These four cytokines activate the HPA axis at the hypothalamic, pituitary, and adrenal level. The receptors for the cytokines are found on hypothalamic neuroendocrine cells and on pituitary corticotrophs (Strand, 1999). These factors are known to cause swelling and inflammation of the surrounding tissue and play an important role in the acute phase response to infection. Elevated levels of IL-1, IL-6 and TNF-α are present in the CNS in a number of neurodegenerative diseases such as Alzheimer’s disease (AD) and Parkinson’s disease (PD). Current evidence suggests that the elevated levels of IL-1, IL-6 and TNF-α in the senile plaques of AD and neurochemical lesions of PD are important factors in the development and progression of the disease state. These increased levels of cytokines may cause a self-propagating inflammatory process contributing to senile plaque formation and disease progression.

The interleukin-1 (IL-1) family of proteins currently comprises IL-1α, IL-1β and the IL-1 receptor antagonist, IL-1ra (O’Neill, 1994). The interleukins IL-1α and IL-1β are synthesized as precursors without leader sequences. The molecular weight of each precursor is 31 kDa (Dinarello, 1997). IL-1α and IL-1β are processed to their “mature” forms of 17 kDa molecular weight polypeptides by specific cellular proteases. The precursor of IL-1α, (proIL-1α), can be processed in one of two ways. ProIL-1α can be cleaved by extracellular proteases after cell death, or cleaved following activation of the calcium dependent, membrane-associated cysteine proteases known as calpains. The
precursor of IL-1β (proIL-1β) on the other hand is processed to its mature form by caspase 1, also called IL-1β converting enzyme (ICE). The final member of the family, IL-1ra, has two isoforms. One is secreted while the second is intracellular. They act as competitive IL-1 antagonists, preventing both IL-1 agonists from binding the IL-1 receptors, inhibiting their biological activity. The primary amino acid homology of mature human IL-1β to IL-1ra is 26%, which is greater than that between IL-1α and IL-1β (Dinarello, 1997).

There are currently two IL-1 receptors recognized. The type I (IL-1RI) is the major receptor on the cell surface, binding both isoforms of IL-1, and transduces a signal to the cell nucleus via activation of the nuclear transcription factor NF κB (Vitkovic, 2000). It is a 80-kDa high-affinity receptor found on several cell types. It works in conjunction in some cell types with the IL-1 receptor accessory protein (IL-1R AcP), which has a molecular mass of 66 kDa. The IL-1R AcP increases the binding affinity of both IL-1β and IL-1β but does not specifically bind either IL-1 agonist. In the immune system, the type I IL-1 receptor is predominantly expressed on T cells and fibroblasts, whereas the type II IL-1 receptor is found on B cells and macrophages (Dantzer, 1997). It has been shown that the type I IL-1 receptor mediates IL-1β-induced fever. The type II IL-1 receptor does not transduce a signal when bound by an active ligand. It therefore acts as a decoy receptor for IL-1 and can negatively regulate the biological activity of IL-1 along with IL-1ra.

Interleukin-6 (IL-6) is a cytokine with a variety of biological functions and can function as either a chemokine or an inflammatory cytokine. It plays a critical role in B cell differentiation, T-cell activation and is a key mediator of the acute phase response (Akira, 1993). Among its other actions IL-6 acts as an inhibitor of TNF-α, another
important inflammatory cytokine induced by lipopolysaccharide (LPS). IL-6 acts as a component of the negative feedback for TNF-α. IL-6 has a wide range of functions in the CNS and in certain disease states. The molecular mass of human IL-6 ranges from 21-28 kDa and is 58% identical to rat IL-6.

IL-6 binds to its receptor to form the biologically active IL-6 receptor complex (IL-6rc), which is made up of two membrane glycoproteins and IL-6. The two membrane glycoproteins are the ligand binding α-subunit (IL-6r or gp80) and the non-ligand binding, signal transducing β-receptor subunit (gp130). The gp130 glycoprotein cannot bind IL-6 itself but it confers high affinity binding and is responsible for the signal transduction. Several other cytokines and hormones share the gp130 subunit as a component for signal transduction. The activation of gp130 allows the JAK family of enzymes to phosphorylate the tyrosine residues of the intracellular part of the gp130 subunit.

Tumor necrosis factor-α (TNF-α) is an important inflammatory mediator with many biological functions. It plays a vital function in the immune system. When released, TNF-α stimulates an increase in the number of B cells, initiation of leukocyte adherence to vascular endothelial cells, and stimulation of other mononuclear phagocytes to secrete IL-1, IL-6 or IL-8 (Prescott, 1996). It can act on the hypothalamus to induce fever and is cytotoxic to tumor cells.

TNF-α has two transmembrane receptors of 55 kDa (TNF-r1) and 75 kDa (TNF-r2). TNF-r1 is the major receptor for TNF-α and binding of TNF-α to the TNF-r1 leads to apoptosis, tissue necrosis, and activation of nonspecific immunity. The TNF-r2 has been implicated in several biological activities including T cell cytotoxicity, thymocyte growth, and the stimulation of granulocyte colony-stimulating factor secretion.
Interleukin-1β Signal Transduction Pathways

The binding of IL-1β to the IL-1R forms the IL-1R AcP complex. This complex activates a cascade of kinases leading to the activation of the transcriptional factors NF-κB and AP-1. The first step in this process is the association of the IL-1 receptor associating kinase (IRAK) with the IL-1R AcP figure (4). IRAK does this by forming a complex with the adapter protein MyD88 and the Tollip protein. Upon stimulation with IL-1 MyD88 recruits the IRAK/Tollip complex to the IL-1RI complex. IRAK is then phosphorylated and released from the IL-1RI complex. The phosphorylated IRAK subsequently interacts with TRAF6 a member of the TNF receptor-associated factor family (Daun, 2000). TRAF6 subsequently associates with the mitogen-activated protein (MAP) kinase complex (TAK1/TAB1) giving rise to a bifurcation of the IL-1 signaling pathway leading to NF-κB and AP-1 activation.

To activate NF-κB the TRAF6/TAK1/TAB1 complex stimulates the NF-κB-inducing kinase (NIK). NIK forms a complex with two kinases, IKKα and IKKβ. IKKα and IKKβ then phosphorylates I-κB, releasing NF-κB which is translocated to the nucleus and activates transcription of κB binding site. In a separate pathway the evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) can interact with TRAF6 to activate the processing of MEKK1. The processed MEKK1 can now activate the IKKα and IKKβ. IKKα and IKKβ, which then follows the same pathway as described above.

To activate AP-1 the TRAF6/TAK1/TAB1 complex activates the MEK3/MEK6 complex. The MEK3/MEK6 then phosphorylates the p38 mitogen-activated protein (MAP) kinase. This kinase then activates c-JUN which is translocated to the nucleus and activates transcription of AP-1. In a separate pathway ECSIT can interact with TRAF6 to activate the processing of MEKK1. The processed MEKK1 can now activate the
MEKK3/MEKK6 complex, which then follows the same pathway as described above.

In a separate pathway independent of MyD88 and IRAK the second messenger diacylglycerol (DAG) is produced by the breakdown of phosphatidylcholine (PC) by a phosphatidylcholine-specific phospholipase C (PC-PLC). Intracellular DAG activates protein kinase C (PKC). The exact role of this pathway is not known at this time, however there is some evidence that this pathway may negatively regulate IL-6 synthesis.

In a third, separate, pathway IL-1β stimulates phospholipase A$_2$ (PLA$_2$) activation to hydrolyze phosphatidylcholine, forming lysophosphatidylcholine-18 (LPC-18) and arachidonic acid (AA). LPC induces the release of IL-6 from the C6 glioma cell line. This induction of IL-6 suggests that LPC acts as a second messenger of IL-1β in the C6 cell line.

Somatostatin

Somatostatin (SRIF) is a multifunctional hormone inhibiting the release of many pituitary, pancreatic, and gut hormones, as well as the exocrine secretions of the gastrointestinal tract (Strand, 1999). It mainly exerts an inhibitory effect on endocrine and exocrine secretion and on the response of various cell types to endocrine stimulation (Ferone, 1999). It inhibits the release of growth hormone (GH), thyroid stimulating hormone (TSH) and prolactin (PRL) by the anterior pituitary. There are two forms of human somatostatin, SRIF-14 and SRIF-28. The primary function of SRIF is inhibitory and acts as an anti-inflammatory substance. There is evidence that SRIF may act as a feedback mechanism in the immune system by regulating the degree of tissue inflammation. In Alzheimer’s disease (AD) the levels of both SRIF and its receptors (especially high-affinity type 1) are decreased with the largest reduction in areas most
affected by AD. The degree of cognitive impairment has been correlated to the loss of SRIF and its receptors.

In rats and humans, SRIF is derived from a single gene to form preprosomatostatin. This is then processed in the cell to form either SRIF-14 or SRIF-28. The two forms of SRIF are stored in separate compartments in the cell until released. The SRIF-28 can be reprocessed to form SRIF-14 if needed, the bond between the 13th and 14th amino acid is broken to form a single protein of SRIF-14. The ratio of SRIF-14 to SRIF-28 found in the cell is approximately 3:1, but SRIF-28 is 3 to 8 times more potent than SRIF-14 in the inhibition of the release of GH. SRIF-14 shows a slightly higher binding affinity for the receptor subtypes 1-4 while SRIF-28 shows a 10 fold higher affinity for the subtype 5 receptor (Benali, 2000). The inhibitor effects of SRIF-28 are generally more potent for a number of biological end points.

There are five transmembrane G protein-coupled receptor subtypes that bind SRIF-14 and SRIF-28 with high affinity. The subtypes 1 and 2 are distributed throughout most of the CNS. Subtype 3 is found in the motor neurons of the brainstem. In the C6 glioma cell line the major SRIF receptor expressed is the subtype 2 receptor, while the subtype 5 receptor is not expressed. All five identified SRIF-receptors can stimulate phosphatidylinositol metabolism, are coupled to inhibitory G proteins, and may inhibit adenylyl cyclase (Akbar, 1994). The five receptor subtypes are 42-60% identical to each other. They are distributed in all the major brain regions, with the highest concentrations in the cerebral cortex, intermediate concentrations in the hypothalamus, thalamus, amygdala, and hippocampus, and the lowest concentrations in the mid- and hindbrain (Strand, 1999). Once SRIF binds to its receptor it inhibits adenylyl cyclase, Ca²⁺ fluxes and hyperpolarizes cell membranes by opening various K⁺ channels. It has been shown
that the SRIF receptor subtypes have unique tissue distributions.

γ-Aminobutyric Acid

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter located in the CNS. GABA is found primarily in the CNS with little or none located in any other body tissue. It is one of several amino acids that play a part in regulating the nervous system. GABA is formed from glutamic acid, one of the traditional amino acids. It mainly exerts an inhibitory effect through its primary receptor, GABA\textsubscript{A}, which forms a functional chloride channel. When activated the receptor causes an increase in the net influx of Cl\textsuperscript{−} ions, which causes hyperpolarization of the neuron. Hyperpolarization of the neuron inhibits the action of excitatory neurotransmitters by raising the threshold for the onset of action potentials.

GABA has two receptor subtypes in the CNS, which have been grouped by their pharmacological action. The GABA\textsubscript{A} receptor-ion channel complex is a heteropentameric glycoprotein of approximately 275 kDa. The receptor complex is composed of five different classes of polypeptide subunits (α, β, γ, δ, and ρ). In addition a number of isoforms have been found so that 15 subunits have been identified. The expression of the subunits for the GABA\textsubscript{A} receptor has been found to vary from one region of the brain to another and confers a diversity of pharmacological responses. The GABA\textsubscript{A} receptor complex forms a Cl\textsuperscript{−} ion channel and has binding sites for benzodiazepines, barbiturates, and a steroid site. Benzodiazepines and barbiturates can allosterically modulate the receptor-channel complex. The GABA\textsubscript{B} receptor is less common in the CNS and is coupled to Ca\textsuperscript{2+} and K\textsuperscript{+} channels. This receptor decreases Ca\textsuperscript{2+} flux and increases K\textsuperscript{+} flux using second messenger systems.
Role of Cytokines in Inflammation and Neurodegeneration

Inflammation is characterized by the migration of leukocytes, increased blood flow and capillary permeability to immune cells to the area of infection or injury. Brain trauma can be caused by a number of factors such as viral infection, neurotoxic injury, loss of oxygen or nutrients and senile plaques caused by Alzheimer disease (AD). The cytokines IL-1β, TNF-α and IFN-γ can increase the damage in the brain when they are not strictly controlled. Cytokines activate glial cells, which in turn produce more cytokines. The resulting brain inflammation can cause the loss of neuronal function.

Current evidence suggests that plaque-associated activated glia and cytokines are important factors in the development and progression of the neuropathological hallmarks of AD. In AD an inflammatory response is mounted in which activated micro- and astroglia and their secreted inflammatory mediators and matrix constituents elicit the transformation of diffuse into neuritic plaques, which secondarily may induce further tissue damage (Heininger, 1999). The activated microglia are found to surround the neuritic plaques in a “halo” effect. The main generators of IL-1 in the brain are activated microglia. In AD patients as compared to age match controls there is a 6-fold increase of activated immunoreactive IL-1 microglia in the CNS. IL-1 expression further correlates with the distribution of neuritic plaques located in the brain. IL-1β has been shown to stimulate the production of IL-6, another cytokine that is important in the inflammatory response and in the progression of AD. Both IL-1 and IL-6 levels in the CNS are increased in the early stages of AD and correlate with the severity of dementia in patients. Further, the amyloid precursor protein (APP) is upregulated by IL-1 and IL-6. The processing of APP to form the β-amyloid protein (βA) that forms the plaques has been linked to activated astrocytes and microglia. Current evidence shows that microglial cells
are directly engaged in the production of the β-amylloid protein. The first deposits of the βA protein appear in the plasma membranes and altered cytoplasmic membranes of microglial cells. Their role in processing the βA is emphasized by the inability to detect the APP mRNA in microglial cells.

AD is a systematic disease that causes alterations in a wide range of systems in the CNS. The GABAergic system has been described as least affected with what appears to be area-specific hippocampal decrements of the GABA_A and GABA_B receptors (Heininger, 1999). The somatostatinergic system shows serious alterations from the norm, SRIF is a multifunctional hormone, which acts primarily as an inhibitory protein. In AD patients the degeneration of somatostatinergic interneurons is present in several CNS locations. This degeneration causes a loss of SRIF-like immunoreactivity and an overall decrease in the SRIF concentrations of the CNS of AD patients. Likewise, there is a decrease in SRIF receptors. The degree of cognitive impairment of AD has been correlated to this loss of SRIF. As will be shown SRIF can inhibit the production of IL-6 by IL-1β. This finding may be of therapeutic value to AD patients in the future.
CHAPTER 2

MATERIALS AND METHODS

Chemicals and Reagents

The 7TD1 hybridoma cell line was obtained from Dr. J. Van Snick, Ludwig Institute (Brussels, Belgium). It is a mouse tumor cell designed to detect the presence of IL-6. The C6 glioma cell line was obtained from the American Tissue Type Culture Collection (Rockville, MD). It is a rat tumor astrocyte cell line used to produce the interleukin-6 protein for detection. Recombinant rat interleukin-1β (IL-1β) was obtained from PeproTech Inc. (Rocky Hill, NJ). Recombinant murine interleukin-6 (IL-6) was obtained from R & D Systems (Minneapolis, MN). (-)-isoproterenol (IPT), (S)-(S)-propranolol (PRO), GABA (γ-amino-n-butyric acid), recombinant somatostatin-14 (SRIF-14), recombinant somatostatin-28 (SRIF-28), isopropanol (reagent grade), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), streptomycin sulfate, penicillin G, gentamycin sulfate and β-mercaptoethanol were obtained from sigma Chemical Co. (St. Louis, MO). 1-capryl-2-hydroxy-sn-glycero-3-phosphocholine (LPC-10), 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC-14), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC-16), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC-18), 1-arachidoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC-20) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). SB203580 and SP600125 were obtained from Calbiochem (San Diego, CA). RPMI-1640, fetal bovine serum (FCS), horse serum
(HS), fungizone and trypsin were obtained from Gibco (Grand Island, NY). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT). Sodium Bicarbonate, sodium phosphate and trypan blue were obtained from Fisher Scientific (Santa Clara, CA).

C6 Glioma Cell Culture

The C6 glioma cells were maintained in continuous culture in a humidified atmosphere of 5% CO₂-95% air at 37 °C in RPMI-1640 medium supplemented with 7.5% horse serum (HS), 2.5% fetal calf serum (FCS) and antibiotics (7.5 μg/mL streptomycin, 15 μg/mL gentamycin, 19 μg/mL penicillin and 0.6 μg/mL fungizone). After 3 days in culture the C6 cells were removed from the tissue culture flask using 0.25% trypsin in phosphate-buffered saline. The C6 cells were then centrifuged at 400 rpm for 5 minutes, the trypsin was removed and the cells were resuspended in the C6 culture medium. The C6 cells were counted using a hemacytometer and were placed either back into continuous culture at 1.0 x 10⁶ cells per 25 cm² flask, or were dispersed into a 96-well tissue culture plates at 1.0 x 10⁵ cells/well with C6 medium for experimental release studies using the 7TD1 bioassay.

C6 cells were allowed to adhere to the plates for 24 hours, after which the cells were rinsed twice with 200 μl serum free RPMI-1640. The C6 cells were then exposed to vehicle (200μL serum free RPMI-1640/well), with and without IL-1β and other agents for 24 hours. When inhibitors were used the cells were pretreated with the appropriate inhibitor for 1 hour in serum free RPMI-1640 and then exposed to the compounds of interest with and without IL-1β. After 24 hours the conditioned media was transferred to a new 96-well plate, which was then stored at 4 °C until measurements for IL-6 by the 7TD1 bioassay.
7TD1 Bioassay for IL-6

The 7TD1 cells were maintained in continuous culture in a humidified atmosphere of 5% CO₂-95% air at 37 °C in 10 mL RPMI-1640 supplemented with 5% FCI (Fetal Clone I; Hyclone, Logan, UT), 50 μM β-mercaptoethanol, 5 pg/mL rmIL-6, and antibiotics. After 3 days in culture the 7TD1 cells were removed from the tissue culture flask using trituration and approximately 1.0 x 10⁶ cells were distributed in a 25 cm² flask (Greiner) for continuous culture, or were dispersed into a 96-well tissue culture plates at 4.0 x 10³ cells/well with SR-RPMI medium for experimental release studies using the 7TD1 bioassay.

We used the IL-6-dependent 7TD1 hybridoma bioassay as described in (MacLeod et al., 1993), with minor modifications to quantify the accumulation of IL-6 in the C6 conditioned media. Conditioned media (20 μL) was cultured in 96 well tissue culture plates in duplicate in 180 μL RPMI-1640 supplemented with 5% FCI, 50 μM β-mercaptoethanol, and antibiotics in the presence of 4,000 7TD1 cells for 72-96 hours. Tetrazolium salt (MTT) was used to determine the growth of the 7TD1 cells. MTT is cleaved in active mitochondria to form a dark blue formazan crystal. After 72-96 hours 20 μL of 5 mg/mL MTT was added to each well for 3.5 hours. 150 μL of medium was removed from each well after the 3.5 hours and the dark blue crystals were dissolved by the addition of 150 μL of 0.04 M HCL/isopropanol to form a dark colored liquid. The plates were stored overnight in the dark after which the optical densities were determined by a microelisa instrument using a test wavelength of 570 nm and a reference wavelength of 630 nm (Dynatech MR5000). For each assay a standard curve of rmIL-6 (0.5 to 64 pg/well) was generated. The data are formed from treatment groups each of which consists of 4 observations per group and are presented as the mean ± SEM.
CHAPTER 3

RESULTS

7TD1 Bioassay Standard Curve

The bioassay standard curves are shown in (fig. 3 and 4). A line-of-best-fit equation was obtained from a dose response curve of rmIL-6 and optical density. In figure, amounts of rmIL-6 greater than 64.0 pg/well do not provide further linear increase in the optical density values. The linear regression equation is easily solved for the x-axis values with a correlation coefficient of >0.99. The minimum amount of IL-6 detectable in this assay was 0.5 pg/well. Samples to be tested need to be diluted properly so that the values generated will fall into the linear portion of the standard curve.

In some cases a logarithmic line-of-best fit equation will generate a better correlation coefficient than a linear regression equation. The values of IL-6 from the logarithmic equation are only calculated on the linear portion of the curve. As with the linear regression equation samples are diluted properly so that the values generated fall into the linear portion of the standard curve. The logarithmic equation is easily solved for the x-axis values with a correlation coefficient of >0.95. The minimum amount of IL-6 detectable in this assay was 0.5 pg/ml. The linear regression equation is used more frequently to generate the line-of-best fit.
Rat Interleukin-1β Dose Response

IL-1β increased the release of IL-6 in a concentration dependent manner (fig. 5). The cells were allowed to incubate for 24 hours. The release of IL-6 by the C6 cells was maximal between 5 and 100 ng/ml IL-1β. For most experiments a concentration of 50 ng/ml IL-1β was used.

Effects of Isoproterenol and IL-1β on IL-6 Release

Isoproterenol (IPT) is a β-adrenergic receptor agonist that activates adenylyl cyclase to increase cAMP concentrations mimicking the actions of norepinephrine. (Fig. 6) shows that there was a 3-fold induction of IL-6 release from the C6 cells when IPT (10 μM) was added to IL-1β (5 to 100 ng/ml). For example, at IL-1β (50 ng/ml) a concentration of 470 ± 45 pg/well of IL-6 was measured. In the presence of IPT and IL-1β there was a synergistic increase of IL-6 release of 1592 ± 104 pg/well. This effect can be blocked by the use of propranalol (PRO), which antagonizes the IPT binding to the β-adrenergic receptor.

Somatostatin Effect on 7TD1 Cell Line

To rule out the possibility that SRIF has any negative effects on the 7TD1 cell line itself, SRIF-14 and -28 were added separately to the 7TD1 bioassay standard curve and compared to a normal standard curve (fig. 7). The concentration of SRIF was calculated as the amount that would be present in the 7TD1 bioassay experiment when a maximum concentration of 200 nM was used in the C6 cell line. All three standard curves were similar suggesting that SRIF has no negative effects on the 7TD1 cell line itself.
Inhibition of IL-6 Induction by Somatostatin

Somatostatin (SRIF) has two forms SRIF-14 and SRIF-28. SRIF is multifunctional hormone, inhibiting the release of many pituitary, pancreatic, and gut hormones. The primary function of SRIF is inhibitory and acts as an anti-inflammatory substance by inhibiting adenylyl cyclase. We decided to investigate the ability of SRIF-14 and -28 to inhibit the IL-1β induction of IL-6 release. In similar SRIF dose response experiments, both forms of SRIF are shown to inhibit the release of IL-6 by IL-1β in the C6 cell line in vitro (fig. 8 and 11). Further both SRIF-14 and SRIF-28 inhibited the synergistic release of IL-6 by IL-1β and IPT (fig. 10 and 13). Both forms of SRIF showed a maximum inhibition of IL-6 at 200 nM. SRIF-14 and -28 appears to possess similar IC_{50} for the inhibition of IL-6 in the C6 cell line in vitro (fig. 14).

γ-Aminobutyric acid Effect on 7TD1 Cell Line

To rule out the possibility that GABA has any negative effects on the 7TD1 cell line itself, GABA was added to the 7TD1 bioassay standard curve and compared to a normal standard curve (fig. 15). The concentration of GABA was calculated as the amount that would be present in the 7TD1 bioassay experiment when a maximum concentration of 1000 nM was used in the C6 cell line. Both standard curves show a very close homology that suggests that GABA has no negative effects on the 7TD1 cell line itself.

Inhibition of IL-6 Induction by γ-Aminobutyric acid

γ-Aminobutyric acid (GABA) is an inhibitory neurotransmitter located in the CNS. GABA is found primarily in the CNS with little or none located in any other body tissue. We decided to investigate GABA ability to inhibit the release of IL-6 by IL-1β. As

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shown in (fig. 16) GABA inhibited the basal and IL-1β stimulated release of IL-6. Further GABA is shown to inhibit the synergistic release of IL-6 by IL-1β and the β-adrenergic receptor agonist IPT (fig. 18).

Proposed Second Messenger of IL-1β

Lysophosphatidylcholine-18 (LPC-18) is a proposed second messenger of IL-1β in the C6 cell line. A number of different chain length forms of LPC-18, which is the natural form, were used. As shown in (fig. 19), LPC induces the production of IL-6 from the C6 cell line in vitro. As with IL-1β, LPC-18 and IPT together will produce a synergistic release of IL-6 (fig. 20). This increased release can be inhibited by the addition of SRIF-14 shown in (fig. 21).

SB203580 Effect on 7TD1 Cell line

To rule out the possibility that SB203580 has any negative effects on the 7TD1 cell line itself, SB203580 was added to the 7TD1 bioassay standard curve and compared to a normal standard curve (fig. 22). The concentration of SB203580 was calculated as the amount that would be present in the 7TD1 bioassay experiment when a maximum concentration of 10 uM was used in the C6 cell line. Both standard curves show that SB203580 does have an effect on the 7TD1 cell line but not enough to account for the large reduction shown in figures 24-27.

IL-1β Signal Transduction Pathways

To better understand the signal pathways of IL-1β that results in the release of IL-6 we used the inhibitors SP600125 and SB203580. SB203580 is a p38 MAPK inhibitor.
that targets the p38α and p38β2 kinases, while the p38β kinase exhibits only partially sensitivity. The inhibitor SP600125 targets the JNK kinase. As shown in (fig. 24), the SB203580 kinase inhibitor resulted in the reduction of IL-6 below detectable levels both basally and with the addition of IL-1β. SP600125 on the other hand showed no inhibition of the release of IL-6 (fig. 25).

To gain further understanding of the signal pathway of LPC-18, we used the proposed second messenger LPC-18 and the p38 kinase inhibitor SB203580 as shown in (fig. 26). In a similar effect seen when used with IL-β, SB203580 resulted in nondetectable levels of IL-6 both basally and in the presence of LPC-18. The SP600125 JNK kinase inhibitor as with IL-1β showed no inhibition of the release of IL-6 when used with LPC-18 (fig. 27).
CHAPTER 4

DISCUSSION

Inhibition of IL-6 Production

We have demonstrated that IL-1β stimulates the release of IL-6 from the rat C6 glioma cell line in vitro. IL-1β also acts synergistically with IPT to increase the release of IL-6. IPT is a β-adrenergic agonist that stimulates the production of cAMP in the cell and thus mimics the effects of the monoamine norepinephrine. The monoamines epinephrine and dopamine have previously been shown to synergistically act with IL-1β to stimulate the release of IL-6, but to a lesser effect than norepinephrine.

IL-6 release is modulated by a number of CNS-acting neurotransmitters. The neurotransmitters SRIF-14, -28 and GABA act primarily as inhibitors in the CNS. SRIF-14 and -28 have five transmembrane G protein-coupled receptor subtypes with distributions in all the major brain regions and a unique tissue distribution. The subtypes 1 and 2 are distributed throughout most of the CNS while subtype 3 is found in the motor neurons of the brainstem. In the C6 rat glioma cell line the major SRIF receptor expressed is the subtype 2 receptor, while the subtype 5 receptor is not expressed. The subtypes 1-4 show a slightly increased affinity for SRIF-14 while subtype 5 shows a 10 fold higher affinity for SRIF-28 (Benali, 2000).

This study demonstrates that IL-6 release from the C6 glioma cell line can be inhibited by neurotransmitters that reduce the intracellular levels of cAMP. Using a
maximal concentration of 50 ng/ml of IL-1β, the levels of IL-6 produced were significantly inhibited by the addition of 200 nM of SRIF-14 or SRIF-28. SRIF-14 inhibited the release of IL-6 to basal levels in the presence of a maximal dose of IL-1β. In contrast, SRIF-28 showed a 65% reduction of IL-6 release from C6 rat glioma cells. Further IL-1β + IPT produces a synergistic production of IL-6. SRIF-14 reduced this production by 82% while in contrast SRIF-28 showed a 43% inhibition of IL-6. It appears that SRIF-14 is a slightly more efficacious inhibitor than SRIF-28 in the C6 rat glioma cell line.

The neurotransmitter GABA opens a functional chloride channel when bound to its receptor. GABA inhibits the actions of excitatory neurotransmitters by raising the threshold for the onset of action potentials for cells by increasing the net influx of Cl− ions. GABA inhibited the production of IL-6 induced by a maximal concentration of 50 ng/ml IL-1β: 1 mM GABA reduced the stimulated release of IL-6 by 85%. The synergistic release of IL-6 by IL-1β and IPT is inhibited by 1 mM GABA by 55%.

To further elucidate the signaling pathway of IL-1β and LPC we used the inhibitors SP600125 and SB203580. SP600125 inhibits the JNK kinase that is postulated to be involved in the release of IL-6. The SB203580 is a p38 MAPK kinase inhibitor that selectively targets the p38α and p38β2 kinases, but has only minimal affects on the p38β kinase. Results show that the SB203580 inhibitor blocked the IL-1β and LPC-18 stimulation of the IL-6 release from C6 rat glioma cells. This indicates that the P38 MAP kinase is an essential component of the intracellular signaling pathway activated by either IL-1β or LPC-18. The SP600125 inhibitor showed no reduction in the levels of IL-6 released from the C6 cell line. This shows that the JNK kinase is not involved in the IL-1β or LPC-18 signaling pathway as previously thought.
LPC-18 is a proposed second messenger of IL-1β. LPC-18 can induce the release of IL-6 from the C6 cell line and IL-1β stimulates the production of this lysophospholipid. LPC-18 is formed by the breakdown of phosphatidylcholine by phospholipase A2 after IL-1β binds to its receptor. The addition of LPC-18 resulted in a similar but lesser release of IL-6 from the C6 cell line compared to IL-1β. LPC-18 mimicked the actions of IL-1β in many aspects; its stimulation of IL-6 release was inhibited by the same neurotransmitters (SRIF-14, -28 and GABA) that inhibited the IL-1β stimulated release of IL-6. Further, the SB203580 and SP600125 kinase inhibitors resulted in the same actions when used in conjunction with IL-1β. SP600125 resulted in no reduction in IL-6 release while SB203580 reduced IL-6 release often to nondetectable levels.

The release of IL-6 is an important facet of the immune system and regulation of the neuroendocrine system. Its release has ramifications on a number of factors in the CNS. When its release is not properly regulated as in AD and other neurodegenerative disorders it can cause damage to surrounding tissue and further the progression of the disease. This study shows that IL-6 release can be regulated by a number of neurotransmitters and inhibitors. Because somatostatin reduction is one of the hallmarks of AD, its ability to regulate IL-6 release suggests that its reintroduction into the CNS of AD patients may prove to have therapeutic value in slowing down the progression of Alzheimer’s disease.
APPENDIX I

LIST OF FIGURES
EXPERIMENTAL PROCEDURE

Day 1
Plate C6 cells for a total volume 200 µL with 1.0 x 10^5 cells/well

Day 2
Wash cells twice
Add drugs, final volume 200 µL

Day 3
Transfer media to new plate and store in the refrigerator

Day 6
Set up plates for duplicates of experiment, concentrated and dilute
Add 180 µL SF-RPMI to concentrated wells
Add 150 µL SR-RPMI to dilute wells
Add 20 µL C6 media to concentrated wells, total volume 200 µL
Discard 50 µL media from concentrated wells
Add 50 µL media from concentrated wells to new set of wells for dilution, total volume 200 µL
Discard 100 µL media from dilute wells
Plate 100 µL 7TD1 cells for a final total volume of 200 µL with 4.0 x 10^3 cells/well

Day 9 - 10
Add 20 µL MTT to each well
Wait 3.5 hours
Remove 150 µL media
Add 150 µL 0.4 M HCL/Isopropanol
Store plates in dark overnight

Day 10 - 11
Read plates

Figure 1: General Schematic for the IL-6 Bioassay. General layout of the experimental procedure used for the production and quantification of IL-6 using the C6 mouse tumour cell line and the 7TD1 rat tumour glial cell line.
Figure 2: Interaction between a neuropeptide, the first messenger, with the surface receptor, and the subsequent conformational change in the receptor that activates a membrane-bound intermediary, a G protein. The G protein activates membrane-bound enzymes that catalyze the production of cytosolic second messengers to regulate a wide variety of cell functions. In many cases, there is signal transduction cross talk between the different second-messenger systems. PIP₂ (phosphatidylinositol 4,5-biphosphate), ATP (adenosine triphosphate), ADP (adenosine diphosphate), CAL (calmodulin), DAG (diacylglycerol), IP₃ (inositol trisphosphate) and cAMP (cyclic adenosine monophosphate).
Figure 3: 7TD1 cell bioassay standard curve with a linear regression line of best fit equation. 7TD1 cells were cultured for 72-96 hours in the presence of increasing amounts of rmIL-6 (.5 to 64 pg/well). The optical density values were obtained with MTT, as described in text. The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 4: 7TD1 cell bioassay standard curve with a logarithmic line-of-best fit equation. 7TD1 cells were cultured for 72-96 hours in the presence of increasing amounts of rmIL-6 (1 to 64 pg/well). The optical density values were obtained with MTT, as described in text. The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 5: Effects of IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI) and IL-1β (0 to 200 ng/ml). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 6: Effects of IL-1β, Isoproterenol and Propranolol on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (0 to 100 ng/ml), IL-1β (0 to 100 ng/ml) + Isoproterenol (IPT) (10 μM) and IL-1β (0 to 100 ng/ml) + Isoproterenol (IPT) (10 μM) + Propranolol (PRO) (10 μM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.

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Figure 7: Effects of Somatostatin-14, Somatostatin-28 and IL-6 on 7TD1 cell bioassay standard curve. 7TD1 cells (8,000 cells/well) were incubated in the presence of 1X-7TD1 media, IL-6 (0.25 to 32 pg/well), Somatostatin-14 (SRIF-14) (200 nM) + IL-6 (0.25 to 32 pg/well) and Somatostatin-28 (SRIF-28) (200 nM) + IL-6 (0.25 to 32 pg/well). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 8: Effects of Somatostatin-14 and IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (100 ng/ml) and IL-1β (100 ng/ml) + Somatostatin-14 (SRIF-14) (0 to 200 nM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 9: Effects of Somatostatin-14 and IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), Somatostatin-14 (SRIF-14) (200 nM), IL-1β (0 to 50 ng/ml) and IL-1β (0 to 50 ng/ml) + Somatostatin-14 (SRIF-14) (200 nM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 10: Effects of Somatostatin-14, IPT, PRO and IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10³ cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (100 ng/ml), IL-1β (100 ng/ml) + IPT (10 uM), IL-1β (100 ng/ml) + IPT (10 uM) + PRO (10 uM), IL-1β (100 ng/ml) + SRIF-14 (200 nM) and IL-1β (100 ng/ml) + IPT (10 uM) + SRIF-14 (2000 nM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 11: Effects of Somatostatin-28 and IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (50 ng/ml) and IL-1β (50 ng/ml) + Somatostatin-28 (SRIF-28) (0 to 200 nM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 12: Effects of Somatostatin-28 and IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), Somatostatin-28 (SRIF-28) (200 nM), IL-1β (0 to 50 ng/ml) and IL-1β (0 to 50 ng/ml) + SRIF-28 (200 nM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 13: Effects of Somatostatin-28, IPT, PRO and IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10³ cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (50 ng/ml), IL-1β (50 ng/ml) + IPT (10 uM), IL-1β (50 ng/ml) + IPT (10 uM) + PRO (10 uM), IL-1β (50 ng/ml) + SRIF-28 (200 nM) and IL-1β (50 ng/ml) + IPT (10 uM) + SRIF-28 (200 nM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 14: Effects of Somatostatin-14, Somatostatin-28 and IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (50 ng/ml), Somatostatin-14 (SRIF-14) (0 to 200 nM) + IL-1β (50 ng/ml) and Somatostatin-28 (SRIF-28) (0 to 200 nM) + IL-1β (50 ng/ml). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 15: Effects of γ-amino-n-butyric acid (GABA) and IL-6 on 7TD1 cell bioassay standard curve. 7TD1 cells (8,000 cells/well) were incubated in the presence of 1X-7TD1 media, IL-6 (0.5 to 64 pg/well) and IL-6 (0.5 to 64 pg/well) + GABA (50 μM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 16: Effects of IL-1β and γ-amino-n-butyric acid (GABA) on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI) = 555.24 +/- 50.89, IL-1β (50 ng/ml) = 1898.999 +/- 132.08, IL-1β (50 ng/ml) + γ-amino-n-butyric acid (GABA) (0 - 1000 μM) and γ-amino-n-butyric acid (GABA) (1000 μM) = 127.72 +/- 34.47. The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 17: Effects of IL-1β and γ-aminobutyric acid (GABA) on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (0 to 25 ng/ml) and IL-1β (0 to 25 ng/ml) + γ-aminobutyric acid (GABA) (1000 µM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 18: Effects of γ-aminobutyric acid (GABA), IPT, PRO and IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10⁵ cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (50 ng/ml), IL-1β (50 ng/ml) + IPT (10 μM), IL-1β (50 ng/ml) + IPT (10 μM) + PRO (10 μM), IL-1β (50 ng/ml) + GABA (1000 nM) and IL-1β (50 ng/ml) + IPT (10 μM) + GABA (1000 μM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.

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Figure 19: Effects of varying types of Lysophosphatidylcholine (LPC 10 to 20) and IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10^5 cells/well) were incubated in the presence of vehicle (SF-RPMI); IL-6 = 105.51 +/- 14.19, varying types of LPC (10 to 20) (0.625 to 40 μM) and IL-1β (50 ng/ml); IL-6 = 1228.45 +/- 35.41. The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 20: Effects of IL-1β, Isoproterenol, Propranolol and LPC-18 on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), IPT (10 uM), PRO (10 uM), IL-1β (50 ng/ml), IL-1β (50 ng/ml) + IPT (10 uM), IL-1β (50 ng/ml) + IPT (10 uM) + PRO (10 uM), LPC-18 (0 to 40 uM), LPC-18 (0 to 40 uM) + IPT (10 uM) and LPC-18 (0 to 40 uM) + IPT (10 uM) + PRO (10 uM). The data are from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 21: Effects of LPC-18, IPT, PRO and SRIF-14 on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), LPC-18 (20 uM), LPC-18 (20 uM) + IPT (10 uM), LPC-18 (20 uM) + IPT (10 uM) + PRO (10 uM), LPC-18 (20 uM) + SRIF-14 (200 nM) and LPC-18 (20 uM) + IPT (10 uM) + SRIF-14 (200 nM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 22: Effects of SB203580 and IL-6 on 7TD1 cell bioassay standard curve. 7TD1 cells (8,000 cells/well) were incubated in the presence of 1X-7TD1 media, IL-6 (0.25 to 32 pg/well) and IL-6 (0.25 to 32 pg/well) + SB203580 (10 uM/well). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 23: Effects of SB203580, IL-1β and LPC-18 on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (50 ng/ml), LPC-18 (40 uM), LPC-18 (40 uM) + SB203580 (0 to 10 uM) and IL-1β (50 ng/ml) + SB203580 (0 to 10 uM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 24: Effects of SB203580 and IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (0 to 50 ng/ml) and SB203580 (10 uM) + IL-1β (0 to 50 ng/ml). The data are formed from treatment groups each of which consists of 4 observations per group and are presented as the mean ± SEM.
Figure 25: Effects of SB203580, SP600125 and IL-1β on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (0 to 50 ng/ml), SB203580 (10 μM) + IL-1β (0 to 50 ng/ml) and SP600125 (10 μM) + IL-1β (0 to 50 ng/ml). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.

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Figure 26: Effects of SB203580, IL-1β and LPC-18 on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), IL-1β (50 ng/ml), LPC-18 (0 to 40 uM) and LPC-18 (0 to 40 uM) + SB203580 (10 uM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.

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Figure 27: Effects of SB203580, SP600125, IL-1β and LPC-18 on IL-6 release from C6 glioma cells. C6 cells (100 x 10^3 cells/well) were incubated in the presence of vehicle (SF-RPMI), SB203580 (10 uM), SP600125 (10 uM), IL-1β (50 ng/ml), LPC-18 (0 to 40 uM), LPC-18 (0 to 40 uM) + SB203580 (10 uM) and LPC-18 (0 to 40 uM) + SP600125 (10 uM). The data are formed from treatment groups incubated for 24 hours each of which consists of 4 observations per group and are presented as the mean ± SEM.

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Figure 28: Possible signaling pathways resulting in IL-6 release and inhibition from C6 glioma cells. AA, arachidonic acid; AdCyc, adenylyl cyclase; ATF2, transcription factor ATF2; β-AdR, β-adrenergic receptor; cJun NH2-terminal kinase; GABA, γ-aminobutyric acid; IL-1β, interleukin-1β; IL-6, interleukin-6, IPT, Isoproterenol (increases cAMP levels); LPC, lysophosphatidylcholine; MAPK, mitogen-activated protein kinases; NE, norepinephrine; NFκB, nuclear factor κB; PC, phosphatidylcholine; PKC, protein kinase C; PLA2, phospholipase A2; PRO, propranolol; SAPK, stress-activated protein kinase; SRIF, somatostatin (reduces cAMP levels).
REFERENCES


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