ROLE OF PAROXETINE IN INFLAMMATORY CYTOKINE RESPONSES IN MACROPHAGES

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ABSTRACT

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G-protein coupled receptors (GPCR) play a critical role in immunity by regulating the function of immune cells including macrophages. Along with their role in GPCR desensitization, G-protein receptor kinases (GRKs) exhibit GPCR-independent roles in modulating various intracellular signaling pathways that regulate inflammatory responses. Paroxetine, a FDA-approved selective serotonin (5HT) re-uptake inhibitor (SSRI) used as an anti-depressant, selectively inhibits GRK2 in cardiomyocytes. We hypothesized that paroxetine inhibits GRK2 activity and affects LPS-induced inflammatory cytokine production in macrophages. Our results revealed that paroxetine decreases LPS-induced IL-6, IL-1β and increases TNFα levels in macrophages. To further evaluate if paroxetine mediated inflammatory cytokine response is through its ability to inhibit GRK2, RNA interference studies were performed with GRK2 siRNA smartpool. These experiments demonstrated that the effects of paroxetine on macrophages are GRK2-independent. Another SSRI, fluoxetine was also found to exert similar LPS-induced differential regulation of cytokine responses in macrophages. However, exogenous 5HT did not affect the inflammatory cytokine response in LPS-induced macrophages. Furthermore, paroxetine did not appear to modulate the following inflammatory signaling kinases in macrophages: pIκBα, pp105, pP38, pJNK1/2, and pERK1/2. A logical continuation of this study would be attempts to elucidate the intracellular pathways involved in SSRI-mediated molecular mechanisms that govern inflammatory cytokine response in macrophages.

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CHAPTER I

AN INTRODUCTION TO G-PROTEIN COUPLED RECEPTOR KINASE 2 AND SELECTIVE SEROTONIN REUPTAKE INHIBITORS IN MACROPHAGES

Introduction

Modulation of inflammatory responses using small molecules represents a viable option for intervention of conditions marked by excessive inflammation. Here, we investigate the role of paroxetine, a selective serotonin reuptake inhibitor (SSRI), in modulating inflammatory cytokine production possibly by affecting G-protein coupled receptor kinase 2 (GRK2) activity in macrophages (Mϕs). Our results indicate that differential regulation of proinflammatory cytokine response in macrophages is likely mediated by a GRK2-independent pathway. In the subsequent sections, topics required for understanding the research questions posed and to reconcile the results of this study with the existing literature are reviewed.

Literature review

Inflammation

Inflammation is the reaction of a vascularized living tissue to an insult that could be biological, chemical or physical in nature. The cardinal signs of inflammation, as first described by Aulus Cornelius Celsus**,** are *rubor* (redness), *tumor* (swelling), *calor* (heat/temperature changes), and *dolor* (pain). Later, Galen added *function laesa* (loss of function) as an additional cardinal sign of inflammation. Inflammation can be acute or chronic in nature depending upon the cause. Acute inflammation is caused due to trauma/injury or a microbial pathogen. Chronic inflammation often ensues acute inflammation in cases of persistent bacterial or viral infection, or could be the result of dysregulated inflammatory response [1]. Recently, disturbances in the metabolic homeostasis have also been identified as chronic inflammation.

As inflammation is the first line of defense against invading pathogens, it is considered as a key component of innate immune response. Key cellular players in inflammation include

Mϕs, neutrophils, dendritic cells, natural killer cells, T-cells, B-cells and other non-immune cells depending on the site of inflammation. These cells detect the presence of pathogens using pattern recognition receptors (PRRs) that act as cognate receptors for a myriad of ligands referred to as pathogen-associated molecular patterns (PAMPs). Mϕs and neutrophils phagocytose the pathogens to contain them at the initial site of infection and are usually destroyed within the phagolysosome, unless the pathogen possesses specific virulence attributes that are capable of subverting this process [2]. Various cytokines and chemokines, molecules that mediate inflammatory response, are produced from these cells upon activation by sensing PAMPs. In addition to cytokines and chemokines, complement system and coagulation factors are also involved in orchestrating a tightly regulated inflammatory and vascular response aimed at clearing the pathogen.

In summary, inflammation is an integral part of innate immunity. However, severe and persistent insult results in the activation adaptive immunity, which is specific to the etiological agent [3,4]. Hallmarks of adaptive immune response include the production of antibodies against pathogen-specific antigens by B cells and cell-mediated immunity by T cells. Mϕ and dendritic cells act as antigen presenting cells to B and T cells and are therefore instrumental in activating adaptive immune response. Innate and adaptive immune responses complement each other in clearing pathogens, rebuilding tissues and ultimately restoring homeostasis [4]. Mϕs which are key players in innate immunity and development of robust adaptive immunity are the primary focus of the research presented here**.**

Macrophages

Macrophages (Mϕ) are a heterogenous group of cells, originally described in the context of their phagocytic capacity by a renowned zoologist, Elie Metchnikoff (http://nobelprize.org/nobel_prizes/medicine/laureates/1908/mechnikov-lecture.html). Mϕ are differentiated from the myeloid lineage of hematopoietic stem cells [2]. Upon entry into the bloodstream, monocytes originating from bone marrow exhibit a great degree of plasticity to adapt to the cues encountered in various tissue microenvironments. This results in the development of tissue specific macrophages, such as the Kupffer cells in the liver and microglia in the central nervous system [5]. Monocytes that differentiate into tissue-specific, resident macrophages arise from a distinct subpopulation of circulating monocytes compared to those cells originating in response to an inflammatory or immunologic stimuli [6]. Resident Mϕs perform a wide range of functions including, sculpting the architecture of various organs during development [7]; maintaining homeostasis by acting as sentinel cells; responding to inflammatory and/or immunologic insult and aiding tissue repair [8,9]. Macrophages that are present in the metabolic organs, such as liver, pancreas and adipose tissue, serve to maintain metabolic homeostasis by acting in concert with the other cells during infection [10]. The role of macrophages in various metabolic disorders like diabetes [11,12], obesity [13,14,15], atherosclerosis [16,17] and metabolic disorders [14,18] have been widely studied and characterized.

Resident Mϕs belong to the reticuloendothelial system and serve as professional phagocytes both in acute and chronic inflammation [6]. Mϕs are key effectors in innate immune response and phagocytose the invading pathogens and their components. As a professional antigen-presenting cell, Mϕs play a critical role in adaptive immunity, by activating multiple

subtypes of lymphocytes by elaborating specific cytokines and expressing various cell surface receptors. Mϕs serve as a major link between the innate and adaptive immunity and facilitates maintenance of homeostasis [8,9].

In conjunction with their progenitor cells and resident Mϕs, Mϕs form the mononuclear phagocyte system (MPS) and can be classified based on different parameters. Most commonly used classification system is based on the mechanisms that result in activation of Mϕs. Activation by interferon γ (IFN γ) or through Toll-like receptors (TLRs), also known as classically activated Mϕs, results in M1 Mϕs, that exhibit a proinflammatory phenotype. Conversely, activation by interleukin(IL)- 4 or IL-13, also referred to as alternatively activated Mϕs [19], leads to the differentiation of M2 Mϕs that are characterized by an anti- inflammatory phenotype [20,21] and regulatory macrophages that are not well characterized [22]. Although this type of classification provides us with a clear description about the macrophage subtypes, there are many more subtypes in between the spectrum of classically and alternatively activated types.

Mϕs display a diverse but unique repertoire of cell surface markers like F4/80, CD11b, CD11c, Ly6G, Ly6C, CD80, CD86, and CD163 [23,24] that are harnessed for detection of Mϕs using flow cytometry and immunohistochemistry [25]. Furthermore, subtypes of Mϕs can also be distinguished by exploiting distinct, subtype-specific cell surface receptors [8]. Mϕs are highly dynamic cells with complex intracellular compartments that aid their major functions, such as receptor-mediated endocytosis and phagocytosis. Intracellular organelles found in Mϕs are similar to those of the normal cells and encompasses nucleus, mitochondria, endoplasmic reticulum with ribosomes and lysosomes. Phagocytosis eventually leads to the formation of phagolysosome and ultimately results in the killing of phagocytosed pathogen, unless the

pathogen subverts the events leading to phagosome lysosome fusion or prevents normal maturation of phagolysosome [26,27].

Receptors present on Mϕ cell surface include pattern recognition receptors, chemokine receptors, peptide receptors, growth factor receptors, complement receptors and G-protein coupled receptors [8,20]. These receptors recognize a myriad of endogenous ligands and pathogen-associated molecular patterns and aid in multifaceted roles of Mϕs. Activation of these receptors trigger an array of inflammatory signaling pathways involving multiple kinases like JAK-STAT, IKKs, PI3K, and Akt, eventually activating nuclear factor kappa B (NFκB) [28,29,30] and other transcription factors leading to the production of several mediators of inflammation. Mϕs produce numerous cytokines, such as IL-6, IL-1β, IL-12, IL-18, IL-10, and tumor necrosis factor alpha (TNFα); and chemokines such as monocyte chemotactic protein (MCP-1), macrophage inflammatory protein-1α (MIP-1α). These cytokines and chemokines are critical players in the initiation and orchestration of immune response. Growth and angiogenic factors including transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF), and fibroblast growth factor, also secreted by Mϕs, assists in wound healing and tissue regeneration.

In light of the central role of Mϕs in both innate and adaptive immune responses, Mϕs are used to investigate the molecular mechanisms of inflammation, its players and also to test small molecule inhibitors modulating inflammatory responses. In the work described in this thesis, Mϕs derived from two disparate sources, RAW 264.7 cells, and primary murine peritoneal Mϕs, were employed to examine the mechanisms of LPS-induced cytokine production.

Toll-like receptors

Toll-like receptors (TLRs) are a heterogeneous family of pattern recognition receptors (PRRs) that communicate the presence of pathogens to host cells [31,32]. TLRs have been the subject of extensive investigation in the last decade [33]. TLRs play an important role in the innate immune system by recognizing various pathogen-associated molecular patterns (PAMPs) found in bacteria or viruses, through 13 different types of receptors elucidated in the Table 1-1.

Upon stimulation with their cognate ligands, these receptors trigger an array of signal transduction pathways involving the recruitment of adaptor molecules such as MyD88 and TRIF [34,35]. This leads to an activation cascade involving kinases including the MAPK pathway and IKKs, eventually leading to the upregulation of transcription factors like NFκB, AP-1 and IRAF [36]. The changes in transcription, mediated by these transcription factors, results in the secretion of a number of cytokines, chemokines and various molecular messengers involved in orchestration of innate and adaptive immune responses [37]. Numerous studies have elucidated the diverse and versatile role of various TLRs and their signaling mechanisms during multiple infectious and inflammatory conditions. Recent studies have demonstrated the involvement of TLRs [38] in autoimmunity, intestinal disorders including Inflammatory bowel disease [39], atherosclerosis [40,41], cancer and neuronal disorders [42].

Table 1-1. Characteristics of Toll-like receptors (TLRs)

Subtype	Ligands	Localization
TLR1	Lipoproteins	Cell surface
TLR2	Lipoteichoic acid from Gram-positive bacteria, Pam3CSK	Cell surface
TLR3	Double stranded RNA, Poly I:C	Intracellular endosomal membrane
TLR4	Lipopolysaccharide from Gram-negative bacteria	Cell surface
TLR5	Flagellin	Cell surface
TLR6	Mycoplasmal lipoprotein	Not known
TLR7	Viral single stranded RNA	Intracellular endosomal membrane
TLR8	Viral single stranded RNA	Intracellular endosomal membrane
TLR9	Bacterial CpG DNA	Intracellular endosomal membrane
TLR11	Profilin (*Toxoplasma gondii*)	Intracellular endosomal membrane
TLR12	Profilin (*Toxoplasma gondii*)	Intracellular endosomal membrane

A member of the TLR family that is studied in some of the experiments described in this thesis is TLR4. Lipopolysaccharide (LPS) is an integral component of the outer leaflet of the Gram-negative bacterial cell wall and is the ligand for TLR4 [43]. Co-receptor proteins CD14 [44], MD-2, and TLR4 are located on the cell surface [45]. Activation by LPS leads to oligomerization and recruitment of adaptor proteins via interaction with TIR (Toll-interleukin-1 receptor) domain consisting of five proteins including TIRAP (TIR domain-containing adaptor protein, also known as Mal), MyD88 (Myeloid differentiation primary response gene 88) [46], TRIF (TIR domain-containing adaptor inducing IFN-β), TRAM (TRIF-related adaptor molecule) and SARM (Sterile alpha and HEAT-armadillo motifs-containing protein) [47]. MyD88-dependant downstream signaling pathway involves the activation of kinases IRAK4, TRAF6, PI3K-Akt, IKKs and MAPKs marked by an increase in the NFκB and AP-1-dependent transcription. These pathways culminate in the secretion of proinflammatory cytokines such as IL-6, IL-1β, and TNFα, and chemokines including IL-8, RANTES, and MIP-1α. TLR4 also activates a TRIF-dependant (MyD88-independent) pathway leading to the activation of TBK1 and increases IRF3 and 7-dependent transcription. TRIF-dependant pathway ultimately leads to IFNα and IFNβ production. Since LPS is a major immunostimulant encountered by the host during bacterial infection, we studied the effects of LPS on Mϕs.

G-Protein coupled receptors (GPCRs)

GPCRs are one of the major types of receptors present on the surface of macrophages regulating their function including cell survival, activation leading to the production of inflammatory mediators and chemotaxis regulating their adhesion and migration to the sites of inflammation [48]. GPCRs are characterized by seven transmembrane domains and constitute a large and versatile superfamily of cell surface receptors. GPCRs bind to a myriad of endogenous

and exogenous ligands and facilitate wide range of cellular and physiological functions including regulation of vision, neuronal transmission, and cardiovascular activity. In addition, GPCRs play a critical role in innate and adaptive immunity [49]. A simple scheme divides GPCRs into three different families: Family A, rhodopsin-like family of receptors that include rhodopsin, adenosine and melatonin among others; Family B, secretin-like family consisting of hormone receptors and metabotrophic receptors; and Family C represented by GABA receptors. GPCRs exert their function through uncoupling of heterotrimeric G proteins ($G_{\alpha\beta\gamma}$ subunits) upon activation by an agonist [50].

Binding of an agonist to a GPCR leads to a conformational change and uncoupling of the $G_{\beta\gamma}$ subunits from GDP bound G_{α} subunit. This event regulates downstream effector enzymes including adenylyl cyclases, phospholipases and various ion channels resulting in either an increase or a decrease in the levels of secondary messengers such as cAMP, calcium (cAMP pathway) or phosphoinositides (phosphotidylinositol pathway) [51]. These second messengers in turn activate PKA, PKC, PI3K, and MAPK/ERK pathways leading to a specific cellular response [49,52].

Regulation of GPCR signaling

Due to the critical nature of GPCR signaling, this signaling system is regulated by feedback systems that prevent overstimulation via GPCRs [53]. Agonist-bound receptors are phosphorylated at specific serine/threonine positions in their intracellular C-terminal domain or the third intracellular loop of the transmembrane by a unique group of kinases, GRKs [49,54]. An overview of regulation of GPCR signaling is provided in Fig. 1-1. The phosphorylated sites on the agonist-bound GPCR recruits scaffolding proteins known as Arrestins [55]. Arrestins are

involved in homologous GPCR desensitization and steric hindrance of further coupling of G-proteins, ultimately leading to internalization of GPCRs via clarithrin coated pits [56,57]. Internalized GPCRs are degraded in the endosome and occasionally activate signaling pathways, intracellularly. GPCRs may also be recycled back to the cell surface for further signaling [55]. Working together, GRKs and Arrestins regulate GPCR signaling by silencing the receptor by desensitization, trafficking the receptor for internalization or degradation and most importantly Arrestin-mediated signaling through various downstream kinases.

G-protein receptor kinases (GRKs)

GRKs are group of serine/threonine kinases that phosphorylate agonist-activated GPCR (Fig. 1-1). These kinases were first discovered during studies on understanding the desensitization mechanisms of visual receptor rhodopsin and β-adrenergic receptor [49,58]. Essential characteristics of GRKs include their strong preference for agonist-activated GPCRs, localization of GRKs to the membrane during GPCR activation and phosphorylation of several sites of the activated GPCR. Additionally, the same type of GRK can phosphorylate different GPCRs indicating redundant function. So far seven GRKs have been identified in mammals and are broadly categorized into three different families based on their sequence homology. The rhodopsin kinase or visual kinase family is comprised of rhodopsin kinase (GRK1) and cone opsin kinase (GRK7). β-Adrenergic kinase or GRK2 family includes GRK2 (β-ARK1) and GRK3 (β-ARK2). GRK4 family encompasses GRKs 4, 5 and 6. Additional characteristics of these GRKs (molecular weight and localization) are presented in the Table 1-2.

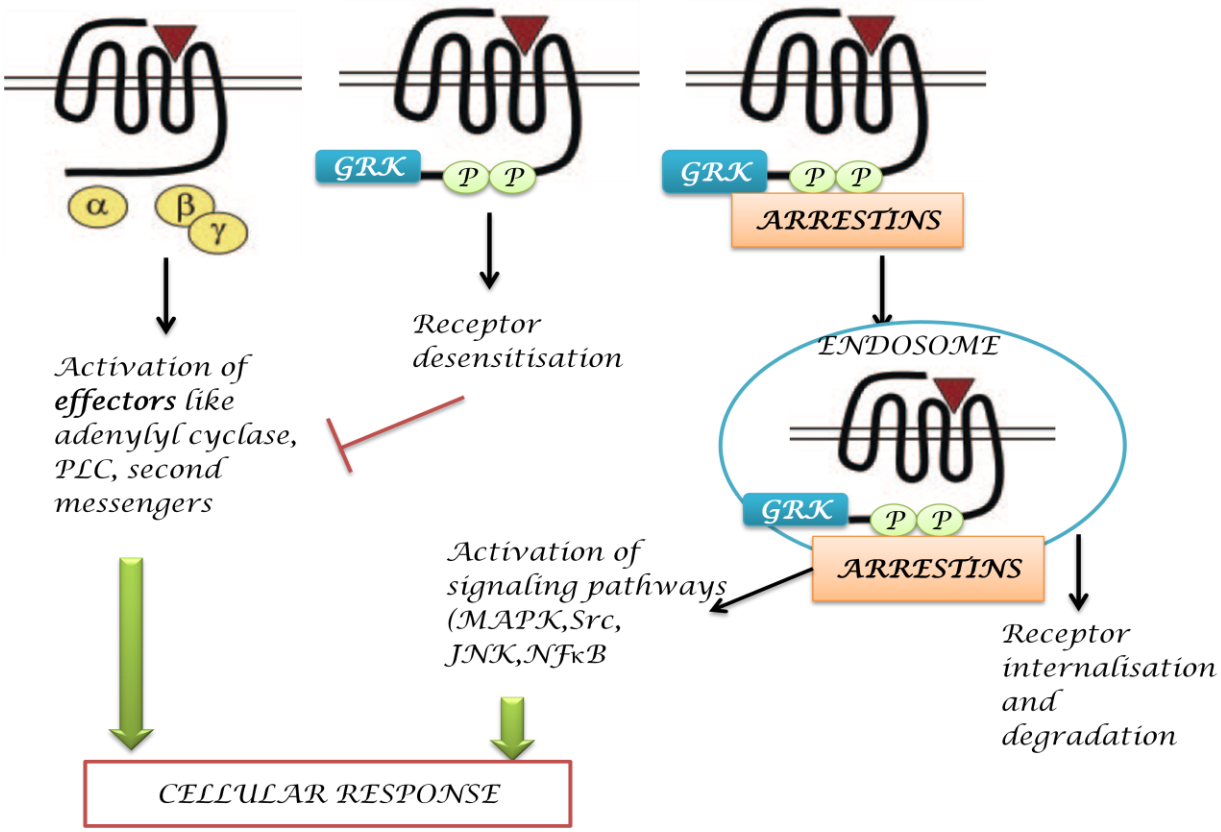

Figure 1-1. G-protein receptor kinase (GRK) dependant G-protein coupled receptor signaling For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.When an agonist binds to GPCR, there occurs uncoupling of G-proteins leading to the activation of various effectors like adenylyl cyclase, PLC, second messengers which lead to essential cellular response. Agonist bound GPCRs are phosphorylated at specific serine/threonine sites in their intracellular carboxy terminal leading to receptor desensitization and cessation of G-protein mediated signal transduction. Phosphorylated GPCRs facilitate the binding of scaffolding proteins called Arrestins in their phosphorylated sites which inturn leads to receptor internalization and degradation in the endosome. Moreover, GRKs and Arrestins induce various intracellular signaling kinases like Mitogen-activated Protein

Figure 1-1 (cont'd) kinases (MAPK), c-Jun-N-terminal kinases (JNK), leading to the activation of Nuclear Factor kappa-light-chain enhancer of activated B cells (NFκB).

Table 1-2. Features of GRKs

GRK	Size (kDa)	Distribution
GRK1 (Rhodopsin kinase)	63	Retina
GRK2	79	Ubiquitous
GRK3	80	Ubiquitous
GRK4	66	Testis, Brain, Proximal tubule of the kidneys, uterine myometrium
GRK5	68	Ubiquitous
GRK6	66	Ubiquitous
GRK7 (Cone kinase)	62	Retina

Structure of GRKs

Seven isoforms of GRKs share a similar tripartite modular structure containing a well conserved central catalytic domain flanked by N-terminal and C-terminal domains. N-terminal domain (183-188 amino acids) of the GRKs includes a region of regulators of G-protein signaling (RGS) domain and it is essential for receptor activation and anchoring on the intracellular side of the cell membrane [53]. The central catalytic domain contains an ATP-binding site and is similar to other serine/threonine kinases. The C-terminal is highly variable across different GRKs and determines the subcellular localization and translocation to the membranes during substrate activation. This domain also has motifs that aid in the interaction of GRKs with phospholipids (PL) and membrane proteins. GRK1 and GRK7 are isoprenylated in their C-termini and are always associated with the cell membrane. GRK2 and GRK3 have an extended C-terminal domain known as pleckstrin homology domain (PH domain) that interacts with PL and G proteins. GRK4 and GRK6 reveal palmitoylation sites and are membrane-associated. GRK5 carries several positively charged amino acid clusters in the C-terminus that aid in binding to PL [49].

Regulation of GRKs

Since GRKs are critical regulators of GPCR signaling, these proteins are subject to regulation by various factors. PIP2(Phosphotidylinositol,4,5 bisphosphate) and Gβγ subunit are the major determinants of GRK activation and membrane localization [53,59]. Although all GRKs contain a PIP2 domain, their affinity and binding position is variable. Binding of PIP2 to GRKs 2, 4, 5 and 6 enhances their phosphorylation and translocation efficiency. However, only

GRKs 2 and 3 contain Gβγ binding domains in their C-terminal domain and this facilitates GPCR-mediated allosteric activation by increasing their kinase activity.

GRKs are also regulated by the intracellular levels of calcium ions (Ca^{2+}). For instance, increased Ca^{2+} levels result in the activation of Protein kinase C (PKC) and thereby simultaneously activates and inhibits GRK2 and GRK5, respectively [60]. Protein kinase A (PKA) activates GRK2 by enhancing its ability to bind Gβγ subunits resulting in increased kinase activity [61]. C-src, a tyrosine kinase directly phosphorylates GRK2 at its tyrosine residues leading to an increase in its catalytic activity. Extracellular signal regulated kinase 1 (ERK1) phosphorylates GRK2 at serine 670, which is present in the Gβγ binding domain, and inhibits its interaction with Gβγ.

Modulation in intracellular Ca^{2+} levels could activate calcium sensor proteins (CSP) [62] , including calmodulin (CaM) [63,64] , neuron-specific calcium sensor proteins known as neuronal calcium sensors (NCS) such as recoverin, visin-like protein (VILIP), neurocalcin, hippocalcin, and S100 family of proteins [62,65]. Calmodulin is the principal regulator of cytosolic Ca^{2+} levels and exerts its activity on GRKs in its Ca^{2+}-bound form. CaM possesses different affinity and sensitivity towards various GRK isoforms. GRK5 is highly sensitive to Ca^{2+}-bound CaM, whereas GRK2 and GRK3 are affected only at higher concentrations. GRK4 and GRK6 are also strongly inhibited by CaM. Recoverin binds directly to GRK1 and inhibits its kinase activity when Ca^{2+} levels are high in the cytosol. NCS proteins have been demonstrated to selectively inhibit GRK1. Recoverin and other NCS are not known in regulating other GRKs.

Caveolin is an integral membrane protein found in the caveolae, the cholesterol and glycosphingolipid-enriched sections of plasma membrane. Caveolin is an additional player feeding into the complex regulatory network that controls the activity of GRKs. It interacts with GRKs and modulates their activity. Binding of caveolin to GRK2 caveolin-binding motifs in their PH domain and N-terminal domain inhibits its kinase activity. Caveolin also inhibits GRKs 3 and 5 via binding to caveolin-binding motifs in the N-terminal domain and controls the basal activity of these kinases [66]. α-actinin belongs to the spectrin superfamily of actin crosslinking proteins. Multiple studies have demonstrated the modulation and/or complete inhibition of the kinase activity of all seven GRKs during interaction with α-actinin.

Physiological roles of GRKs

Understanding the mechanism and activity of GRK isoforms through targeted deletion and/or overexpression has provided greater insight into their role in the regulation of signal transduction through GPCRs. GRK1 deficiency leads to prolonged response of the rhodopsin receptor resulting in light-induced apoptosis in rod cells [67]. GRK2 plays an important role in cardiac development and function. Homozygous GRK2 knockouts in mouse are embryonically lethal around day 9-12 indicating the importance of this kinase in the normal development and tissue homeostasis. Variations in the deficiency of GRK2 affected the phenotype differently [68,69]. Recently, impact of endothelial GRK2 on vascular homeostasis has been demonstrated. GRK2-HDAC6 interaction was found to modulate cell spreading and motility [70]. GRK3 deficient mice have a normal embryonic and post-natal development except for the loss of olfactory receptor desensitization [71]. Genetic depletion of GRK4 in mice, found mainly in the reproductive organs of both male and female, did not affect fertility. Overexpression of GRK4 was found to regulate dopamine-1 receptor activity in kidney inturn regulating essential

hypertension [72,73]. GRK5 deletion caused a rise in the cholinergic responses such as hypothermia, salivation, hypoactivity and antinociception. M2 muscarinic receptor desensitization was found to be GRK5-dependant [74,75]. GRK6 plays an important role in the desensitization of various chemokine receptors of immune cells. Loss of GRK6 results in decreased chemotaxis of lymphocytes but enhanced bone-marrow derived neutrophils in the circulation during inflammation [76,77].

GRKs and pathological conditions

Due to the inherent biological importance of GPCR signaling, aberrant levels of GRKs lead to disturbances in the tissue homeostasis. Mutation in GRK1 results in type 2 Oguchi disease characterized by congenital stationary night blindness [78,79]. GRK2 levels were shown to be increased in chronic cardiac failure [69,80], left ventricular disorders [81] and cystic fibrosis [82]. However, GRK2 levels are lower in rheumatoid arthritis and opiate-addiction related disorders. GRK3 is found to regulate corticotrophin release hormone receptor type I signaling [83]. A single-nucleotide polymorphism in GRK3 has been implicated in bipolar disorder [84]. GRK4 has been implicated in essential as well as salt-induced hypertension [73], and in ovarian cancer [85]. Role of GRK5 in pathologic cardiomyopathies has been extensively studied. GRK5 modulates growth of prostrate tumor [86]and early stage Alzheimer's-like pathology [87,88]. Deficiency or mutation in GRK6 is marked by an autoimmune disease due to impaired apoptosis in mice [89] and is also associated with increased metastasis of medulloblastoma [90,91].

GRKs in inflammation

GRKs 2, 3, and 5 are highly expressed in immune cells and their levels are regulated during inflammation by various ligands suggesting their involvement in inflammatory disorders and immune activation [58]. Recent literature sheds more light on the role of all isoforms of GRKs in modulating inflammation. The major focus of this thesis project is GRK2 and its role in inflammation is presented here. GRK2 levels were increased in murine peritoneal macrophages when treated with various TLR ligands including LPS, Pam3CSK4 and PolyI:C [28,69]. Previous work in the Parameswaran laboratory has unraveled the role of myeloid-specific GRK2 as a negative regulator of NFκB-p105-ERk pathway, thereby limiting the pathological changes encountered during endotoxemia in mice [29]. In addition to the GPCR-dependent role of GRK2 in inflammation, GRK2 is also attributed with a GPCR-independent role mediated by direct protein-protein interactions in regulating various inflammatory signaling pathways [69,92]**.** Platelet-derived growth factor receptor-β (PDGFR β) is phosphorylated at Ser1104 by GRK2 leading to receptor dimerization and desensitization in vascular smooth muscle cells [93,94]. GRK2 binds tubulin, a cytoskeletal protein, through its C-terminal domain indicating its role in regulating microtubule and cytoskeleton reorganization in HEK293 cells [94][95] . GRK2 interacts with RKIP (Raf kinase inhibitor protein) to blocks its kinase activity, thereby prolonging the desensitization and signaling processes. RKIP belongs to a family of phosphatidylethanolamine-binding proteins (PEBPs) that inhibit various kinase signaling pathways [96].

Direct interaction between GRK2 and MEK1 (Mitogen activated protein kinase1) has been demonstrated and this interaction modulates ERK1 activity [97]. Additionally, functional interaction between GRK2 and proteins such as PI3K (phosphoinositide-3 kinase) [98] , Akt, GIT (GRK interacting protein) [92] and heat shock protein (hsp90) [99] has been demonstrated.

Changes in GRK2 levels are associated with chemotactic disturbances during inflammation. Modulating the signaling pathways affected by GRKs represents an avenue that can be harnessed for pharmacological intervention of inflammation. A recent study has identified paroxetine as a specific inhibitor of GRK2, albeit in the context of cardiac muscle.

Paroxetine

Paroxetine, an FDA-approved drug commonly used to treat depression in humans, was recently identified as a specific inhibitor of GRK2 by Tesmer *et al.* [100] An RNA aptamer displacement assay, specific to inhibiting GRK2 with nanomolar affinity, was performed with ~40,000 compounds. Preliminary screen led to the identification of paroxetine (Paxil™) as a selective inhibitor of GRK2 with ~60-fold selectivity over other GRKs. Thermal stability and ROS phosphorylation assays demonstrated that paroxetine binds directly to GRK2 inhibiting its kinase activity. Specificity to GRK2 was confirmed with a phosphorylation assay using thyrotrophin releasing hormone receptor. Crystallographic and diffraction data suggested that paroxetine inhibits GRK2 by reorganizing its active site. Paroxetine increases myocardial contractility in isolated adult mouse ventricular myocytes *in vitro* and cardiac inotropy in wild-type mice *in vivo*. Taken together, these findings establish paroxetine as a specific inhibitor of GRK2 activity in cardiac myocytes. Paroxetine is a member of Selective Serotonin Reuptake Inhibitors (SSRIs) and SSRIs are used routinely to treat depression. SSRIs include paroxetine (Paxil, Pexeva), fluoxetine (Prozac), citalopram (Celexa), escitalopram (Lexapro), sertraline (Zoloft). Among these molecules, paroxetine is the most potent SSRI and exhibits a half-life ranging from 21 to 32.

Pharmacokinetics

SSRIs are prescribed mainly for major depressive disorder but are also used in the treatment of anxiety disorders, panic disorders, eating disorders and occasionally for post-traumatic stress disorder. SSRIs are administered orally and metabolized through hepatic first pass metabolism by cytochrome P450 system [101]. Paroxetine is absorbed readily from the gastrointestinal tract and metabolized in the liver. Trace amounts of paroxetine are excreted in the feces. Optimal dosage of paroxetene is 20 mg/kg body weight [102]. Adverse effects include nausea, nervousness, agitation, libido, drowsiness, insomnia, headache, xerostomia, emesis and diarrhea.

Mechanism of action of SSRIs

SSRIs were identified based on their ability to inhibit of reuptake of serotonin through serotonin transporters (SERT) on the cell surface of the pre-synaptic neuron in a sodium-dependent manner [103]. Serotonin (5HT) is a monoamine neurotransmitter synthesized from tryptophan, an aromatic amino acid. 5HT is primarily synthesized in gastrointestinal (GI) tract and central nervous system. Non-neuronal serotonin has a different effect compared to the neuronal serotonin in influencing gastric motility and also modulating inflammation. Tryptophan hydroxylase (TPH) is the rate limiting enzyme in 5HT biosynthetic pathway (Fig. 1-2) and catalyzes the production of 5-hydroxy-L-tryptophan (5HTP) from L-tryptophan. Two isoforms of TPH has been described; TPH1 is found in the enterochromaffin cells of the GI tract and produces ~90% of the 5HT, TPH2 is synthesized in the neurons [104]. The second step in the 5HT pathway involves decarboxylation of 5HTP by 5-hydroxytryptophan decarboxylase forming 5-hydroxytryptamine/serotonin. 5HT is metabolized within cells by monoamine oxidases into 5-hydroxy indoleaceticacid (5-HIAA). 5HT released by the presynaptic neuron in the synapse activates 5HT receptors (comprises of seven isoforms) present in the post-synaptic

neuron. Within the synapse, 5HT acts on the SERT aiding in their reuptake in a sodium-dependant manner and 5HT is stored intracellularly confined to vesicles (Fig. 1-2). As a consequence of inhibition of SERT by SSRIs, the level of synaptic 5HT available for signaling increases. 5HT produced in the GI tract is released into the circulation, where it is selectively stored by the platelets. Macrophages and other lymphocytes possess functional TPH1 and these observations suggest that these cells might represent non-traditional sources of 5HT [103].

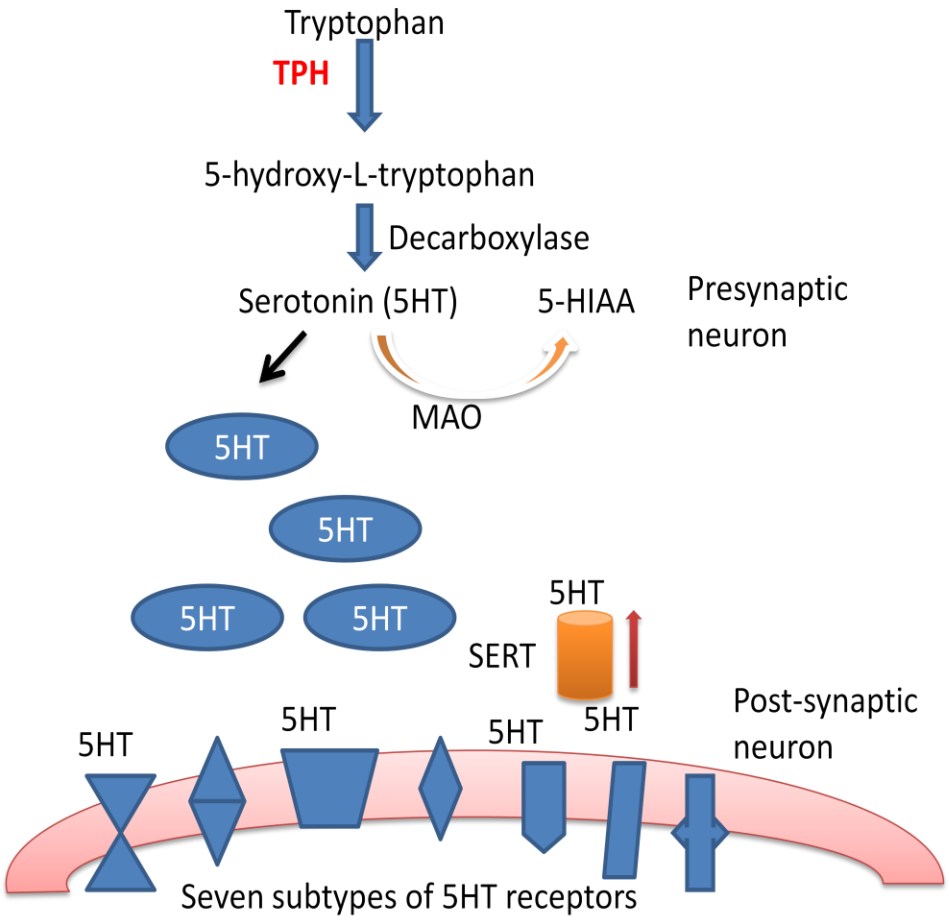

Figure 1-2. Serotonin biosynthesis and transport in pre-synaptic neuron. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.Serotonin is synthesized from tryptophan. Tryptophan hydroxylase (TPH) is the rate limiting enzyme that converts tryptophan to 5-hydroxy-L-tryptophan (5HTP). Decarboxylation of 5HTP by 5HT decarboxylase forms serotonin (5HT) which is stored in vesicles in the pre-synaptic neuron. Upon stimulation, 5HT is released extracellularly into the synapse by exocytosis and acts on seven different subtypes of 5HT receptors on the post synaptic Figure neuron. Moreover, 5HT in the synapse is taken back into the pre-synaptic neuron through serotonin transporters (SERT). 5HT can be metabolized intracellularly by monoamine oxidases (MAO) into its main metabolite 5-hydroxyindoleacetic acid (5-HIAA).

Role of SSRIs in inflammation

Pathophysiology of the major depressive disorder in humans continues to remain elusive. However, various theories have been proposed to unravel the mechanism and to evaluate new therapeutic targets in treating depression. Popular theories include altered neurotransmitter (5HT, dopamine, and norepinephrine) levels in the brain, stress induced functional impairment of the neuroendocrine system, altered levels of tryptophan and dysregulated immune responses [105]. Nevertheless, these theories do not explain the effects and severity of types of inflammation that could result in clinical depression. Recent studies have established neurogenic inflammation as the cause of depression in humans. Studies have also shown that increased pro-inflammatory cytokine (IL-6, IL-1β, and TNFα) levels, both in periphery and in the brain, precipitate development of depression. Although anti-depressants in clinical use are effective in ameliorating the symptoms, there is considerable interest in identification of novel antidepressants and understanding the mechanism of action of existing antidepressants.

SSRIs have been found to posses various anti-inflammatory properties in neuronal and non-neuronal tissues [106,107]. Recently, paroxetine was found to inhibit inflammation in brain and loss of neurons in an experimental model of Parkinson's disease [108]. Fluoxetine and other SSRIs reduced depression and dementia related to multiple sclerosis and Alzhimer's disease. Since these SSRIs possess anti-inflammatory properties, it is possible that they could be used for non-neuronal chronic inflammatory disorders. Paroxetine and fluoxetine were found to decrease pro-inflammatory cytokine levels (IL-6, IL-1β, and TNFα) in animal models of endotoxemia [106,107] that is concomitant with better outcomes for the host. Paroxetine and sertraline were found to improve endothelial function, decrease inflammatory mediators, and improve cardiac function in patients with coronary heart disease [109]. Fluoxetine exhibits anti-inflammatory

effect in a broad spectrum of inflammatory diseases including experimental colitis [110], periodontitis [111] and LPS-induced microglial inflammation. Paroxetine, fluoxetine and amitryptaline were found to ameliorate inflammation in rodent models of adjuvant-induced arthritis [112]. Additionally, SSRIs were also proposed to enhance wound healing due to their potent endothelium-protective and anti-platelet functions [113].

Role of 5HT in inflammation

5HT plays a critical role in modulating gastric motility, epithelial cell secretion and vasodilation [114]. Recent studies have revealed that 5HT receptors are involved in inflammation of the gut [115]. 5HT is shown to play both offensive and defensive roles in maintaining homeostasis in the gut. Inhibitors of TPH1 and multiple 5HT receptor antagonists (5HT7, 5HT3) reduce 5HT-induced bowel inflammation. Platelets release 5HT that is stored in cytoplasmic vesicles during activation. 5HT derived from platelets mediate immune functions by modulating the recruitment of immune cells, including neutrophils, to the site of infection during acute infection, and eosinophil migration into the lung during allergy. Platelet-derived 5HT is also involved in skewing macrophage polarization into M2 phenotype thereby regulating inflammatory reponses [116]. Addition of exogenous 5HT to activated macrophages decreases pro-inflammatory cytokine levels at 72 hr post LPS stimulation. 5HT acts as a pro-inflammatory molecule in adjuvant-induced animal models of arthritis [117]. 5HT was also shown to activate intracellular kinase MAPK ERK pathway in peripheral blood monocytes through 5HT1A receptor [118].

5HT receptor agonists and antagonists and their role in disease models

Given the versatile roles of 5HT in multiple disease conditions of great importance to human health, thorough understanding the mechanism of action of 5HT through its respective receptors might unravel novel use for 5HT and its receptor antagonist as a therapeutic agent for various disorders. 5HT receptor (5HT1, 5HT2, 5HT6, and 5HT7) antagonists are also used in the treatment of depression. 5HT1and 5HT2 receptor agonists have been used as analgesics and for mitigating migraines due to its ability to induce the release of endorphins and substance P. 5HT3 receptor antagonists are used in the treatment of nausea and emesis. 5HT4 receptor agonists are used as gastric prokinetic agents. Recently, 5HT7 receptor antagonist was shown to ameliorate inflammation and increase survival in a dextran sodium sulfate induced experimental model of colitis [119]. 5HT2/5HT7 receptor antagonist, LY215840, delays platelet release and artery occlusion in animal models of thrombosis [120].

Rationale

Mϕ are a key player in both innate and adaptive immune response. Mϕs possess a plethora of cell surface receptors including PRRs and GPCRs that in turn regulates cellular function. GPCRs and GRKs represent potential targets to manipulate Mϕ function using small molecules. Paroxetine, an SSRI, was found to selectively inhibit GRK2 activity in cardiomyocytes. The major question addressed in this thesis is whether paroxetine could be used to modulate inflammation-related signaling events in Mϕs and if this effect is mediated by GRK2 in Mϕs**.**

Central hypothesis for this study was that paroxetine modulates inflammatory responses in Mϕs primarily by inhibiting GRK2 activity. First part of this thesis describes efforts **to**

elucidate the effect of paroxetine on TLR signaling in Mϕs. The role of paroxetine on TLR signaling, specifically TLR4 was investigated using murine macrophage cell line (Raw 264.7 cells) and primary mouse peritoneal macrophages. The second part of this thesis attempts to unravel the mechanisms involved in the effect of paroxetine on LPS-induced inflammatory cytokine responses with an emphasis on its ability to inhibit GRK2 or by modulating 5HT pathways in macrophages. RNA interference was employed to knock-down GRK2 levels in Raw 264.7 cells and the effects on paroxetine on Mϕs in the presence of normal and reduced levels of GRK2 were tested. Regulation of inflammation by paroxetine in Mϕs could be an indirect effect caused by increased 5HT concentration as Mϕs possess tyrosine hydroxylase-1 (TPH-1), the rate-limiting enzyme in 5HT biosynthetic pathway. Different concentrations of 5HT was used to explore the effects of 5HT on LPS induced inflammatory cytokine response. Because of the key role of Mϕs in both innate and adaptive immune responses, understanding and modulating their endotoxin- induced inflammatory cytokine response by molecules might provide a novel treatment strategy for conditions caused by excessive inflammation.

CHAPTER II

MATERIALS AND METHODS

Reagents

Paroxetine hydrochloride hemihydrate (MW 374.83) and Fluoxetine hydrochloride (MW 345.79) were purchased from Sigma-Aldrich (St Louis, MO). Serotonin hydrochloride (M, 217.18) and LY 215840 (MW 400.04) were obtained from Tocris bioscience (Bristol, UK). RPMI 1640 (Rosewell Park Memorial Institute) media, Fetal Bovine Serum (FBS), Pen-strep (Penicillin Streptomycin mixtures contain 5,000 units of penicillin and 5,000 µg of streptomycin/ml in saline) and Versene (0.2 g EDTA/liter of PBS) were purchased from Life technologies (Carlsbad, CA).

Antibodies

Antibodies (P-IκBα, P-ERK, P-JNK, Pp38, Pp105, and tubulin) were purchased from Cell Signaling Technology Inc. (Danvers, MA). Antibodies (GRK2 and ERK2) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Alexa fluor goat anti-rabbit antibody (Invitrogen, Carlsbad, CA) and anti-mouse IgG IRdye 800 conjugated antibody (Rockland Immunochemicals Inc, Gilbertsville, PA) were used with LICOR Odyssey system. Peroxidase conjugated anti-mouse antibody (Vector Laboratories Inc, Burlingame, CA) developed with Immunocruz luminal reagent (Santa Cruz Biotechnology) was used for chemiluminescence.

Animals

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were housed in groups of 4 to 5 mice per cage in rooms maintained at 22-24˚C with 50% humidity with 12 hour light and dark cycle. All animals had access to normal chow and water *ad libitum*. Experimental procedures involving mice were conducted in accordance to the protocol approved by the Institutional Animal Care and Use Committee at Michigan State University.

Cell culture

RAW 264.7 cells (murine leukaemic monocyte macrophage cell line) were derived from the peritoneal exudate of male BALB/c mice infected with the Abelson murine leukemia virus [121,122]. Raw 264.7 cells were purchased from ATCC (Manassas, VA) and grown in RPMI media supplemented with 10% Fetal Bovine serum (FBS) along with 1% PenStrep and maintained in 5% CO_2 at 37˚C. RAW cells were passaged and cells from 10 to 20 passages were used for the experiments.

Primary mouse peritoneal macrophages

Four mice were injected intraperitoneally with 1ml of 4% Brewer's thioglycollate broth (4.05% thioglycollate, Sigma-Aldrich, St Louis). Peritoneal cells from each mouse were collected 4 days post injection, individually, as previously described by Barski's modification in 1995. Mice were euthanized in a CO_2 chamber and a cutaneous vertical midline incision was made in the abdomen. The peritoneal cavity was washed twice with 10ml of RPMI media supplemented with 10% FBS. Peritoneal washes were centrifuged at 1500 RPM for 5 minutes. The pellet was washed with ACK lysis buffer (NH_4Cl 8,024 mg/l, $KHCO_3$ 1,001 mg/l and EDTA Na_22H_2O 3.722 mg/l) to remove the erythrocytes from the cell pellet and centrifuged as mentioned above. This step was repeated once again to ensure complete removal of erythrocytes. 10^6 cells/well were seeded in 12-well plates for the experiments.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants from the treated cells (RAW and peritoneal macrophages) were collected and cytokine analysis for IL-6, IL-1β,TNFα and IL-10 were performed according to manufacturer's instructions using ELISA Kits (eBioscience, San Diego, CA). Optical density

measurements were taken at 450nM in an Infinite® M1000 PRO plate reader (Tecan, Mannedorf, Switzerland). Cytokines were quantified using the Magellan data analysis software (Tecan, Mannedorf, Switzerland) and normalized to the total cellular protein (μg). Protein levels were determined by Bradford assay (Biorad, Hercules, CA)

RNA Interference

Control siRNA (small interfering RNA) pool (against the luciferase gene, neither present nor expressed in macrophages), GRK2 siRNA smart pool (against mouse GRK2), were purchased from Dharmacon (Fisher Scientific, Pittsburgh, PA). Raw 264.7 cells were transfected by electroporation using Amaxa Nucleofector II (Lonza, Cologne, Germany) with the program D-032 as previously described. To cell pellets containing 2×10^{6} cells, 100 μl of the nucleofector solution and 200nM of the corresponding siRNA was added. Contents of the transfection reaction were mixed thoroughly by pipetting, transferred to the special cuvette provided in the kit, and electroporated in the nucleofector using the program D-032. The electroporated cells were then resuspended in 800 μl of the FBS-supplemented RPMI media and distributed equally to 3 wells in 12-well plates. The cells were analysed for knockdown of GRK2 after 48 h following every transfection experiment by western blotting.

Western blot analysis

Treated Raw 264.7 cells were lysed using a lysis buffer cocktail consisting of NP-40 lysis buffer containing complete protease (Roche,) and phosphatase inhibitors. Intact cells and debris from lysed cells were cleared by centrifugation at 13,000 RPM for 10 min at 4° C. Protein concentration in the lysates were determined using Bradford assay (Biorad, Hercules, CA). Equal amounts of protein along with 6X loading dye were electrophoresed in 10% SDS PAGE gels consisting of stacking (4% acrylamide) compartment on top of resolving compartment (10%

acrylamide). Separated proteins were transferred to a nitrocellulose membrane. Membranes were blocked with LICOR blocking buffer for fluorescence imaging or with 5% non-fat dry milk in phosphate buffered saline containing 0.05% tween-20 for horse-radish peroxidase (HRP) conjugated secondary antibodies. Immunoblotting was done by incubating the membranes with primary antibodies followed by secondary antibodies which were either fluorescence dye conjugated that were analysed using LICOR's Odyssey or HRP- conjugated analysed by chemiluminiscence. Densitometric analysis was done using the Odyssey software for fluorescence antibody and ImageJ software was used to evaluate the bands from chemiluminescence blots. Tubulin was used as the endogenous control to normalize protein concentrations.

Statistical analysis

All the experiments were repeated 3 to 6 times and each "N" represent values from a different passage of Raw 264.7 cells and a different mouse in case of peritoneal macrophages. Error bars depicted in the figures correspond to SEM. Data was analyzed using GRAPHPAD PRISM software (San Diego, California, USA). Comparisons between the treatment groups were done using one-way ANOVA with corrections for multiple comparisons with Post-Holm-Sidak test and a P value <0.05 was considered as a statistically significant difference.

CHAPTER III

EFFECTS OF PAROXETINE ON MODULATING INFLAMMATION IN MACROPHAGES

RESULTS

Effect of paroxetine on LPS-activated primary mouse peritoneal macrophages

GRK2 is expressed in high levels in the immune cells[92].Our lab has previously demonstrated that activation of mouse peritoneal macrophages by LPS increases GRK2 expression in a time-dependant manner[28]. Additionally, GRK2 possesses GPCR- independent role in regulating various inflammatory signaling pathways including NFκB in macrophages [123]. Paroxetine, an FDA approved drug, inhibits GRK2 in cardiomyocytes [100]. In the current study, we tested whether paroxetine acts as an inhibitor of GRK2 function in macrophages. Primary peritoneal macrophages were collected as described in Methods and 1×10^6 cells were seeded per well in 12-well plates. Macrophages were treated with two different concentrations of paroxetine hydrochloride (10 μM and 20 μM). Twenty minutes post incubation at 37˚C and 5% CO_2 , LPS (1μg/ml) was added to both paroxetine-treated cells and untreated cells and cells were incubated for another 6 or 24 h. Media supernatants were collected and cytokine concentrations (IL-6, IL-1β and TNFα) were determined using ELISA according to manufacturers' instructions. Consistent with our prediction, LPS-activated peritoneal macrophages produced increased levels of IL-6, IL-1β and TNFα compared to the untreated controls, whose values were below detectable levels (Fig. 3-1). On the other hand, paroxetine-treated macrophages stimulated with LPS exhibited decreased IL-6 and IL-1β levels compared to untreated controls. 20 μM paroxetine resulted in profound decrease in cytokine levels compared to 10 μM paroxetine at both 6 h and 24 h time-points (Fig. 3-1A and 1B). The differences in cytokine levels were statistically significant at 24 h; however, a similar trend marked by reduced cytokine levels were observed at 6 h. LPS stimulation of paroxetine-treated cells resulted in

elevated TNFα levels compared to untreated cells in a dose-dependent manner (Fig. 3-1C). In summary, these results suggest that paroxetine modulates LPS-mediated secretion of inflammatory cytokines from primary murine peritoneal macrophages.

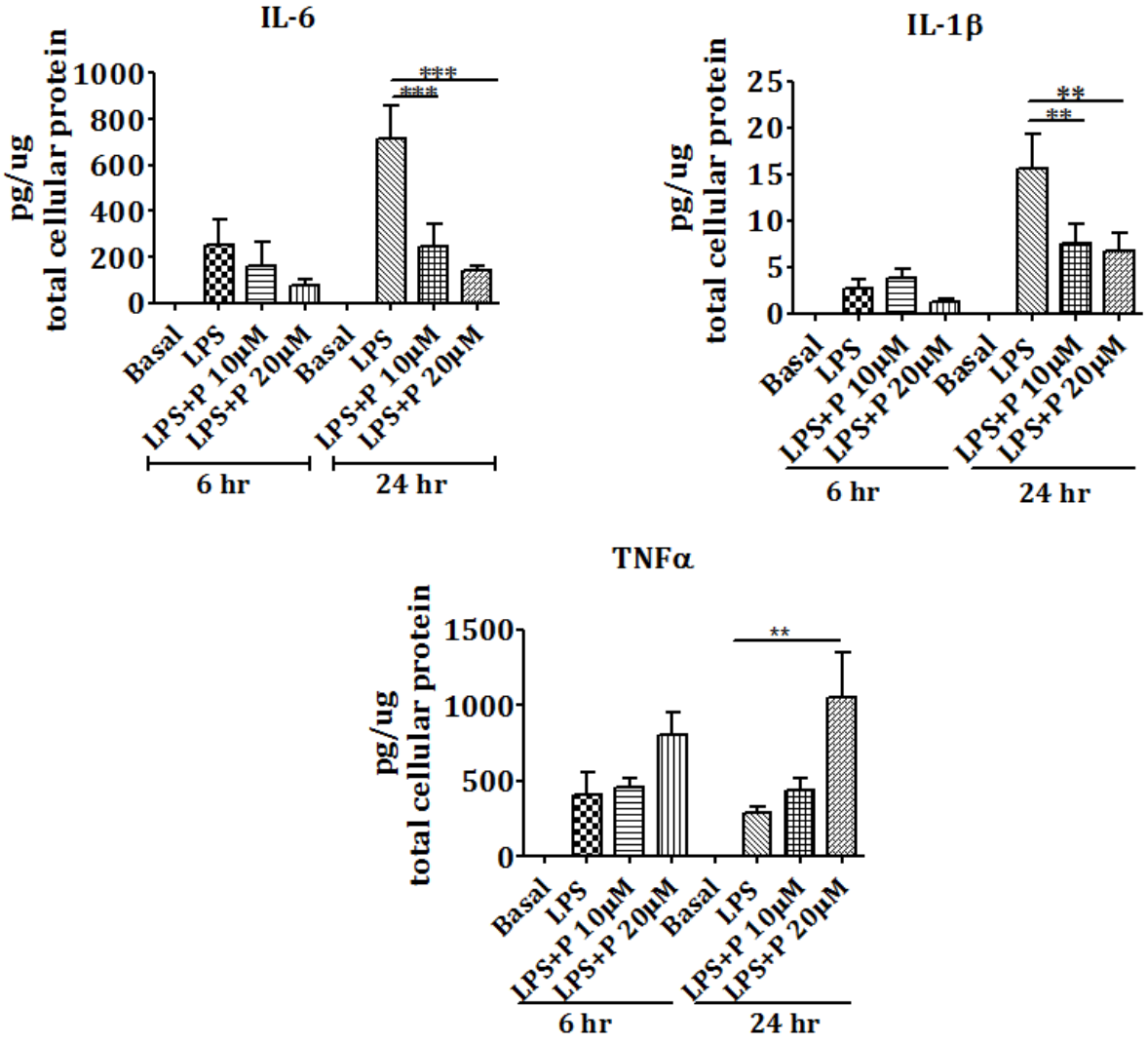

Figure 3-1. Paroxetine modulates secretion of proinflammatory cytokines in mouse peritoneal macrophages: Thioglycollate-elicited peritoneal macrophages were treated with or without LPS (1μg/ml) and paroxetine hydrochloride (10μM, 20μM) for the indicated times. Levels of inflammatory cytokines IL-6 (A), IL-1β (B), TNFα (C) were measured in media supernatants by ELISA. Levels were normalized to total cellular protein and expressed as pg/μg of total cellular protein. Results were analyzed by GRAPHPAD PRISM software. The comparisons between the treatment groups were done using ANOVA with corrections for

Figure 3-1 (cont'd) multiple comparisons along with Post-Holm-Sidak test N=5, ** P=0.0001, ***P<0.0001 and error bars indicate Mean ±SEM.

Paroxetine decreases pro-inflammatory cytokines IL-6 and IL-1β but increases TNFα in RAW 264.7 cells

To verify if the effects of paroxetine are limited to primary peritoneal macrophages, RAW 264.7 cells, a murine leukemic macrophage cell line, was also used in the paroxetine treatment assays. Previously studies [29,124] [125,126] have reported the use of RAW 264.7 cells as a faithful model to investigate LPS-mediated inflammatory responses in macrophages. Experiments were repeated exactly as described for primary mouse peritoneal macrophages. Paroxetine significantly decreased IL-6 levels at both 6 and 24 h at 20μM concentration whereas IL-1β was reduced significantly at 24 h post LPS-stimulation (Fig. 3-2 A and B). Consistent with the observation in peritoneal macrophages, paroxetine increased TNFα levels significantly at both 6 and 24 h at 20μM concentration. These results confirm that the effect of paroxetine on LPS induced IL-6, IL-1β and TNFα is not restricted to primary mouse macrophages and can be replicated in macrophage cell line.

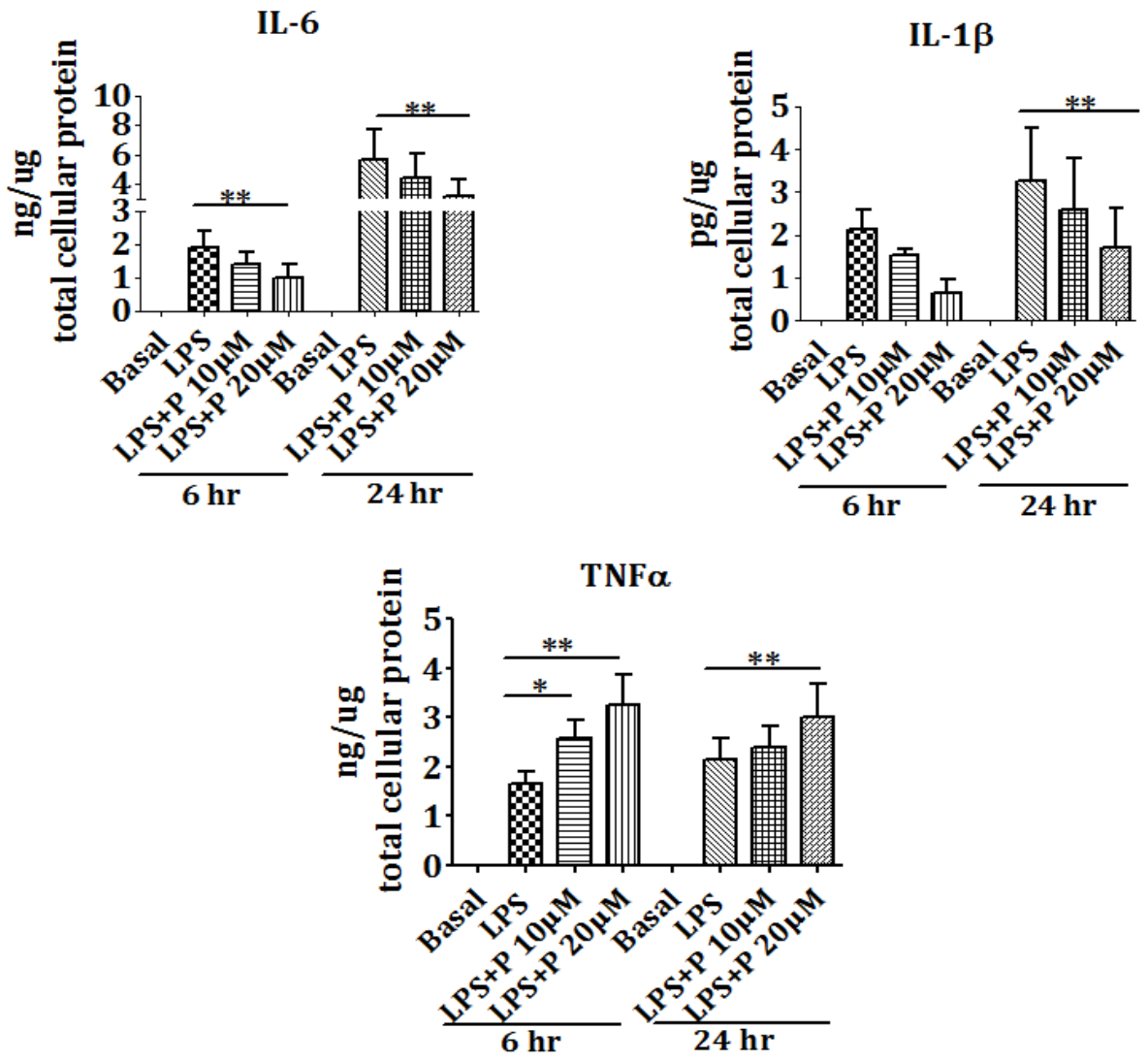

Figure 3-2. Paroxetine decreases IL-6 and IL-1β but increases TNFα in LPS-treated RAW 264.7 cells. RAW 264.7 cells were treated with or without LPS (1μg/ml) and paroxetine hydrochloride (10μM, 20μM) for the indicated time. ELISA was used to determine the levels of proinflammatory cytokines IL-6 (A), IL-1β (B), TNFα (C) in media supernatants. Cytokine levels, normalized to total cellular protein, are expressed as pg/μg or ng/μg of total cellular protein. ANOVA with corrections for multiple comparisons along with Post-Holm-Sidak test

Figure 3-2 (cont'd) was used to compare the treatment groups and analyzed using GRAPHPAD PRISM. Error bars represent Mean ± SEM. N=6, * P=0.02, **P=0.005

Effect of Paroxetine on LPS-induced inflammatory response in reduced levels of GRK2 in macrophages

Tesmer *et al.*, have reported that paroxetine is a selective inhibitor of GRK2 in cardiomyocytes. GRK2 is known to modulate inflammatory response in RAW 264.7 cells by regulating NFκB activity[29]. To investigate whether paroxetine induced inflammatory cytokine response in macrophages are mediated through GRK2, GRK2 knockdown experiments with siRNA smartpool was performed in RAW 264.7cells. Cells were transfected with either control or GRK2 SiRNA from smart pool with amaxa nucleofector. GRK2 knockdown at 48 h post transfection was confirmed using western blotting . Fluorescently labeled secondary antibody was used and the blots were developed with LICOR biosciences Odyssey system as previously described [29]. Consistent knockdown (75±5 %) of GRK2 was achieved in RAW 264.7 cells transfected with GRK2 siRNA compared to control siRNA (Fig. 3-3). 48 h post transfection, cells were treated with paroxetine (20μM). Twenty minutes later, LPS (1μg/ml) was added and cells were incubated for an additional 24 h at 37˚C. Media supernatants were collected and used for measuring inflammatory cytokines (IL-6, IL-1β and TNFα) using ELISA. Levels of IL-6 and TNFα secreted by GRK2 knockdown cells were similar to control cells (Fig.3- 4). IL-1β levels were decreased, but not statistically significant, in GRK2 knockdown cells (Fig. 4). However, paroxetine treatment resulted in reduced IL-6 and IL-1β and increased TNFα levels, irrespective of the GRK2 levels in these cells (Fig. 3-4). Taken together, these results suggest that the effect of paroxetine on LPS mediated inflammatory cytokine response in macrophages is likely GRK2-independent .

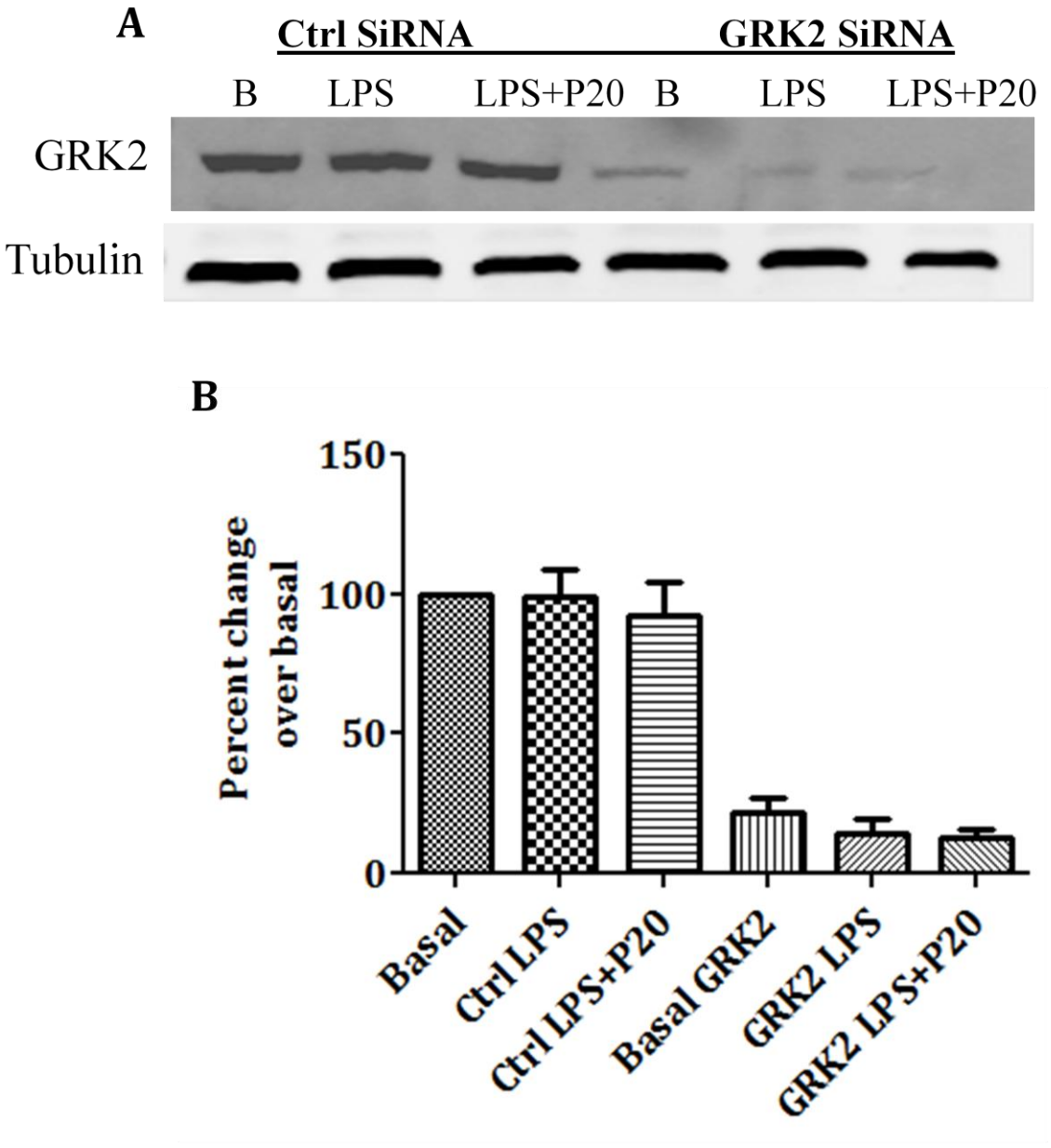

Figure 3-3. Knockdown of GRK2 in RAW 264.7 macrophages using siRNA. RAW 264.7 cells were transfected with either control SiRNA or GRK2 SiRNA smart pool using amaxa nucleofector (Program- D032). 48 h post transfection, cells were treated with paroxetine (20μM) and LPS (1μg/ml). Whole cell lysates, separated on SDS page gels, were transferred to nitrocellulose membranes and probed with anti-GRK2 antibodies. (A) A representative blot depicting the knockdown of GRK2 in cells transfected with GRK2 siRNA compared to control

Figure 3-3 (cont'd) siRNA. Various treatment groups are indicated. Immunoblots were developed using LICOR Odyssey system. Tubulin was used as a loading control. (B) Blots were quantified and normalized to tubulin (N=5) and expressed as percent change over basal level

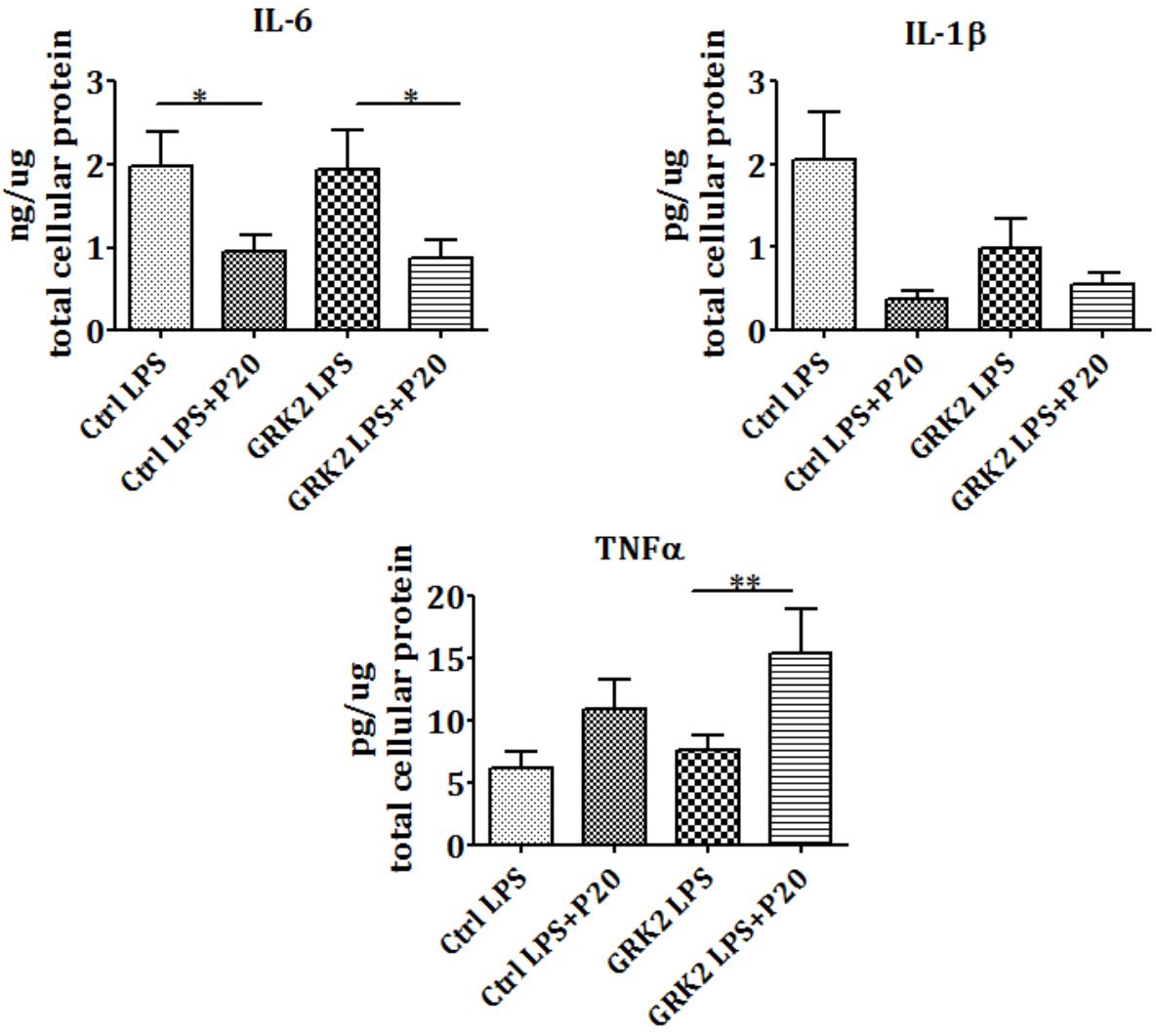

Figure 3-4. Effect of Paroxetine on LPS-induced inflammatory response in reduced levels of GRK2 in macrophages. RAW 264.7 cells were transfected with control and GRK2 SiRNA

Figure 3-4 (cont'd) smartpool. Cells were treated with paroxetine (20μM) and LPS (1μg/ml) at 48 h post transfection. IL-6 (A), IL-1β (B), and TNFα (C) were measured from culture supernatants at 24 h by ELISA. Levels were normalized to the total quantity of cellular protein and expressed as pg/μg or ng/ μg of total cellular protein. Results were analyzed by GRAPHPAD PRISM software (San Diego, California, USA). The comparisons between the treatment groups were done using One-way ANOVA with corrections for multiple comparisons along with Post-Holm-Sidak test and error bars indicate Mean ±SEM. N=5, * P=0.05, ** P=0.002

Fluoxetine also affects cytokine response in LPS-activated macrophages

Our results indicate that effect of paroxetine on LPS-activated macrophages is independent of GRK2. To further confirm the GRK2-independent effect of paroxetine, fluoxetine, an SSRI which is chemically related but structurally unrelated to paroxetine [100] was used. Moreover Fluoxetine did not inhibit GRK2 in cardiomyocytes [100]. Fluoxetine has been previously demonstrated to possess anti-inflammatory effects in mouse models of endotoxemia [107], colitis [110] and periodontitis[111]. To investigate the effects of fluoxetine on LPS-activated macrophages, primary mouse peritoneal macrophages and RAW 264.7 cells were treated with fluoxetine. Experiments were repeated essentially as described for paroxetine treatment assays with fluoxetine (10μM and 20μM). Surprisingly, Fluoxetine decreased IL-6 and IL-1β levels in both the cell types. However, IL-6 levels were significantly different at 24 h in peritoneal macrophages (Fig. 3-5 A) and at both 6 and 24 h in RAW 264.7 cells (Fig.3-6 A). IL-1β levels were significantly different only at 24 h for both cell types (Fig 3-5 B and 3-6 B). Additionally, fluoxetine also increased TNFα levels in both cell types with statistically significant differences observed at 24 h for peritoneal macrophages (Fig. 3-5 C) and at both 6 h and 24 h for RAW 264.7 (Fig.3-6 C). In summary, these results suggest that the effect of paroxetine on proinflammatory cytokine secretion in macrophages is likely GRK2-independent since fluoxetine also exerts comparable effects on LPS-induced inflammatory cytokine response in both types of macrophages.

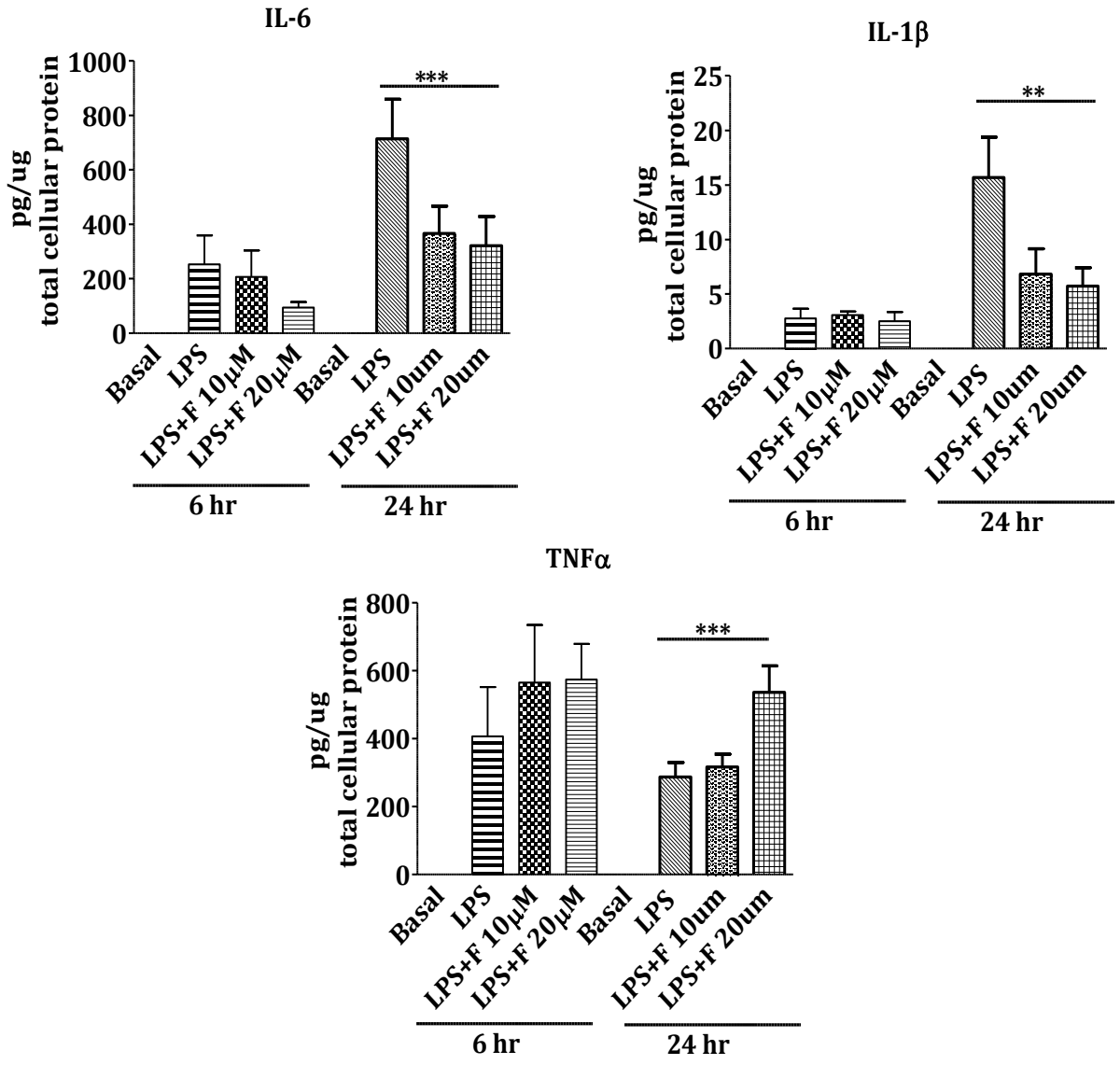

Figure 3-5. Effect of Fluoxetine on LPS-activated mouse peritoneal macrophages

Thioglycollate-elicited peritoneal macrophages were treated with LPS (1μg/ml) and fluoxetine (10μM and 20μM) for the indicated time. Levels of inflammatory cytokines IL-6 (A), IL-1β (B), TNFα (C) were measured in media supernatants by ELISA. Cytokine levels were normalized to total cellular protein and expressed as pg/μg of total cellular protein. Results were analyzed by GRAPHPAD PRISM software (San Diego, California, USA). ANOVA with corrections for

Figure 3-5 (cont'd) multiple comparisons along with Post-Holm-Sidak was used to compare the treatment groups and error bars indicate Mean ±SEM. N=4, ** P=0.007, *** P=0.0002

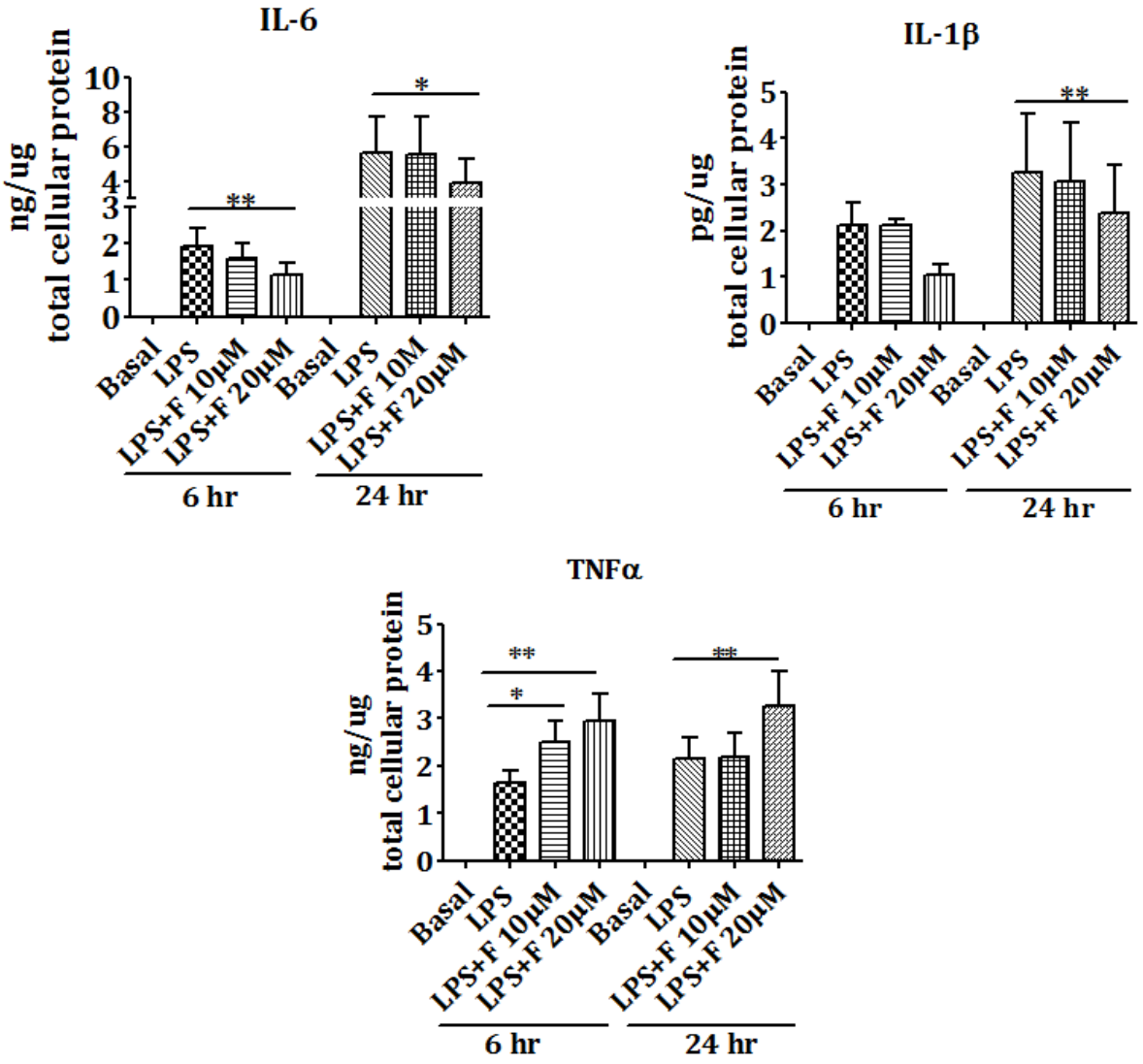

Figure 3-6. Effect of Fluoxetine on cytokine secretion in LPS-activated RAW 264.7 cells

RAW 264.7 cells were treated with LPS (1μg/ml) and paroxetine hydrochloride (10μM and 20μM) for the indicated time. Levels of proinflammatory cytokines IL-6 (A), IL-1β (B), TNFα (C) were measured in media supernatants by ELISA. Cytokine levels were normalized to total

Figure 3-6 (cont'd) cellular protein and expressed as pg/μg or ng/ μg of total cellular protein. Results were analyzed by GRAPHPAD PRISM software . ANOVA with corrections for multiple comparisons along Figure 3-6 (cont'd)- with Post-Holm-Sidak was used to compare the treatment groups and error bars indicate Mean ±SEM. N=6, * P=0.02, ** P=0.006

Effect of serotonin (5HT) on proinflammatory cytokine response in macrophages

Paroxetine and fluoxetine, both members of SSRI family, exert similar effects on the cytokine responses of LPS-activated macrophages. Therefore, it is plausible that the effect of SSRIs on macrophages could be mediated by modulating 5HT levels similar to their well characterized function indispensable for the anti-depressant activity of these compounds. SSRIs inhibit serotonin transporters (SERT) found on the presynaptic neuron, thereby increasing the availability of serotonin (5HT) within a synapse; leading to greater activity of 5HT [103]. Furthermore, macrophages contain TPH1, the rate limiting enzyme in 5HT biosynthesis [115] and 5HT modulates macrophage function through 5HT2/5HT7 receptors[127]. Therefore it is important to evaluate the role of 5HT on cytokine production by LPS-activated macrophages. RAW 264.7 cells were seeded at 1×10^6 cells per well in 12-well plates and incubated overnight at 37˚C and 5% CO_2. Cells were treated with LPS (1μg/ml) and 5HT (100nM, 1μM and 10μM). Under these conditions, LPS-induced inflammatory cytokine response was not influenced by 5HT (Fig. 3-7) in macrophages. Results of the 5HT experiment (Fig.7) suggests the possibility of two scenarios. Endogenous serotonin already released by LPS stimulation exerts max effect therefore masking the effects of exogenous 5HT. Another possibility is that 5HT is not involved in Paroxetine's effects of modulating cytokine secretion in LPS-activated macrophages. This result indicates that enhanced secretion of proinflammatory cytokines in LPS-induced macrophages exposed to paroxetine and fluoxetine is likely independent of 5HT levels.

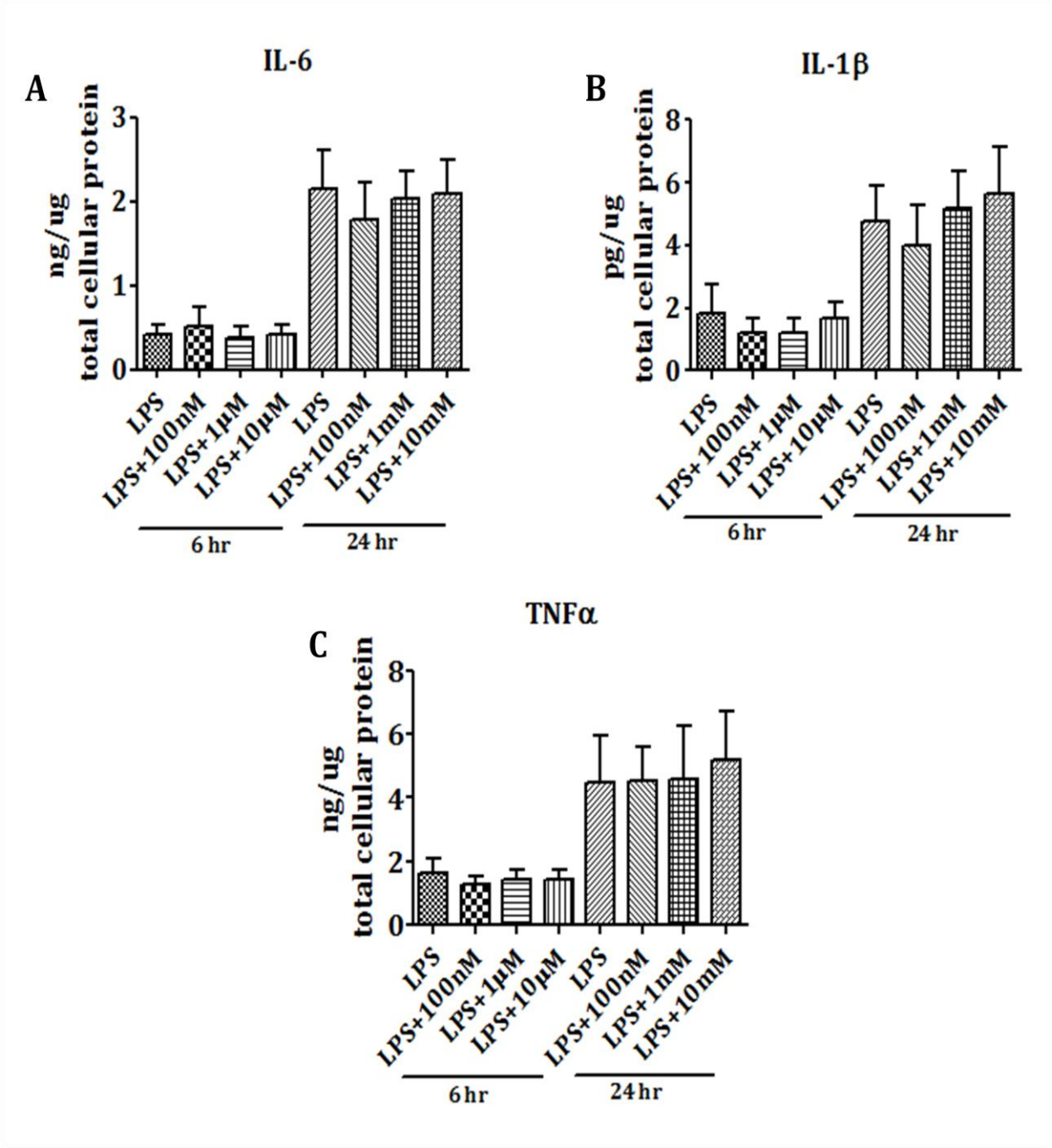

Figure 3-7. 5HT does not affect cytokine secretion in LPS-activated RAW 264.7 cells. RAW 264.7 cells were treated with LPS (1μg/ml) and 5HT (100nM, 1μM and 10μM). Media supernatants were collected and levels of proinflammatory cytokines IL-6 (A), IL-1β (B), TNFα

Figure 3-7 (cont'd) - (C) were measured by ELISA. Levels were normalized to total cellular protein and expressed as pg/μg or ng/ μg of total cellular protein. Data were analysed usin GRAPHPAD PRISM software and the error bars represent Mean± SEM. N=6

Effect of Paroxetine on inflammatory signaling mechanisms in macrophages

Activation of macrophages with LPS is known to modulate various downstream inflammatory signaling pathways including NFκB, a major transcription factor regulating inflammatory cytokine production [130]. Paroxetine could act on a broad range of signaling pathways affecting cytokine response in LPS-activated macrophages. To understand the mechanisms of Paroxetine's effects, we investigated the effects of LPS and paroxetine on various signaling pathways including pIκBα, pp105, pP38, pJNK1/2, pERK1/2 in macrophages.

RAW 264.7 cells were seeded in 6-well plates at a concentration of 2×10^6 cells/well. Cells were treated with LPS (1μg/ml) and paroxetine (20μM) for 30 and 60 minutes. Whole-cell lysates were separated by SDS-PAGE and probed with specific antibodies against pIκBα, pp105, pP38, pJNK1/2, ERK and pERK1/2. Secondary antibodies, either HRP tagged (pIκBα) or IR dye labeled (pp105, pP38, pJNK1/2, pERK1/2,ERK, tubulin), were used to visualize and quantify levels of these proteins. However, paroxetine does not appear to affect the phosphorylated levels of these kinases in macrophages (Fig. 3-8).

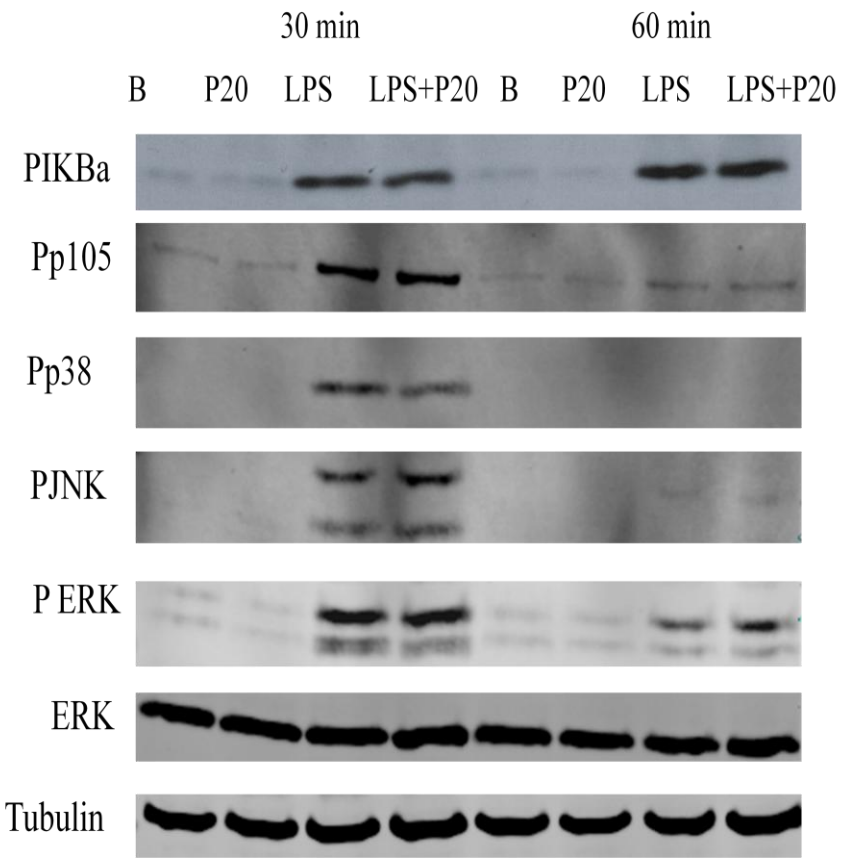

Figure 3-8. Effect of paroxetine on inflammatory signaling mechanisms in macrophages

RAW 264.7 cells were treated with LPS (1μg/ml) and paroxetine (20μM) for 30 and 60 minutes. Whole-cell lysates were collected and separated by SDS PAGE and immunoblotted using various antibodies. Immunoblots for pIκBα, pp105, pP38, pJNK1/2, pERK1/2,ERK and tubulin are depicted here.

CHAPTER IV

DISCUSSION

The major focus of this thesis project is G-protein coupled receptor kinase 2 (GRK2) and its role in inflammatory responses in macrophages (Mϕs). GRK2 is expressed in high levels along with GRK5 and GRK6 in immune cells, so the effects of GRK2 in modulating inflammation is extensively studied and characterized. GRK2 expression levels were altered in various human inflammatory and neurodegenerative diseases including sepsis [131], rheumatoid arthritis [132], multiple sclerosis [133] and Alzheimer's disease [134] in specific cell types. GRK2 levels are regulated by various TLR (Toll-like receptor) agonists that act as primary pathogen-associated molecular patterns (PAMPs) associated with inflammation in a temporal fashion in Mϕs [28] and neutrophils [135]. Previously, GRK2 was shown to interact with various intracellular kinases, phosphorylating and inactivating p38 MAPK in HEK293 cells [136], but acting as a negative regulator of NFκB-p105 ERK pathway in peritoneal Mϕs from myeloid-specific GRK2 knockout mice [123]. Since G-protein coupled receptors (GPCRs) and GRKs have been implicated in various pathological states, modulating their activity using small molecules represents a novel intervention strategy against these conditions. Immense interest exists in targeting kinases as drug targets and is exemplified by the fact that protein kinase inhibitors account for approximately 25% of the pharmaceutical targets [137]. Experiments described in this thesis precisely address this subject by exploring the use of an FDA-approved drug in modulating inflammatory response in Mϕs.

Paroxetine is a FDA-approved drug that belongs to the family of selective serotonin reuptake inhibitors (SSRIs) and is widely used in the treatment of depression in humans, was recently found to selectively inhibit GRK2 in cardiomyocytes. Tesmer *et al.,* determined that paroxetine, by directly binding to the active site of GRK2, inhibits phosphorylation of adrenergic resecptors resulting in enhanced cardiac contractility in cardiomyocytes *in vitro* and *in vivo*

[100]. Since, GRK2 is a major player in determining the inflammatory state of Mϕs [29,123] current work addresses the question of whether paroxetine could act as a GRK2 inhibitor and modulate LPS-induced inflammatory cytokine responses in Mϕs. Primary mouse peritoneal Mϕs and RAW 264.7 cells treated with paroxetine produced lower levels of IL-6 and IL-1β and increased levels of TNFα (Chapter 3. Fig. 3-1 and 3-2). Our data is consistent with another study involving fluoxetine in differentially regulating proinflammatory cytokine production in LPS-activated microglial cells at concentrations (5μM or less) [106] lower than those used in the current study (10 and 20 μM). Paroxetine decreased both TNFα and IL-6 levels in the microglial cells unlike our observation of higher TNFα levels in paroxetine-treated Mϕs.

The differences in cytokine secretion profile of macrophages are possibly arising from cell-specific effects of paroxetine. Although anti-inflammatory properties have been attributed to paroxetine, the molecular mechanisms are yet to be unraveled. Differential regulation of IL-6, IL-1β and TNFα could be explained by considering the differences in the secretion of these cytokines, involving various downstream inflammatory intracellular signaling kinases [138] [126,139,140]. Changes in cytokine levels could be regulated during transcription or translation or post-translationally during secretion. Molecular mechanisms of interaction of paroxetine with the components of transcription and translational machineries might provide more insight into understanding its differential effect. LPS-induced inflammatory cytokine responses in Mϕs are highly dynamic and are controlled by a complex regulatory network.

RNA interference studies revealed a marked decrease in the IL-6 and IL-1β levels and increase in TNFα in the paroxetine-treated GRK2 knockdown cells compared to untreated controls in RAW 264.7 cells (Chapter 3. Fig. 3-3). Our results also indicate that LPS-induced inflammatory cytokine responses were not affected by GRK2 knockdown compared to the

control LPS-treated cells. Previous studies have shown that GRK2 negatively regulates TLR4:LPS signaling in primary cells. Paroxetine exhibits similar effects on cytokine secretion in cells containing reduced levels (75±5 %) of GRK2 compared to cells with normal GRK2 levels. Taken together with the similar effect of fluoxetine, which is not a GRK2 inhibitor (Chapter III, Figure3-5,3-6) our results suggest that Paroxetine likely acts via GRK2 independent pathway. Taken in light of the previous report on paroxetine [100], our results emphasize that cell-specific effects should be carefully considered when exploring the function of a small molecule in any novel cell type or model.

Paroxetine and other SSRIs are the first line anti-depressants in clinical use. In addition to their anti-depressant activity, a number of SSRIs including paroxetine, fluoxetine, sertraline, venflaxine,and fluoxamine have been attributed with anti-inflammatory properties including LPS-activated inflammatory responses in microglial cells [106,107], mouse models of experimental colitis [110], arthritis [112], periodontitis [111] and also in facilitating wound healing [113]. RAW 264.7 cells and primary mouse peritoneal Mϕs treated with fluoxetine revealed similar differential regulation of LPS induced IL-6, IL-1β and TNFα levels (Chapter 3 and Fig. 3-4 and 3-5). Taken in conjunction with the results of paroxetine treatment assays, these results suggest that the effects observed during LPS-induced cytokine response in Mϕs could be due to a shared mechanism. Therefore, it is possible that any SSRI could exert the same effect on cytokine secretion in LPS-induced Mϕs.

As paroxetine and fluoxetine exhibit comparable effects on LPS-induced cytokine production, it is reasonable to speculate that these effects are mediated through their ability to increase 5HT levels. Exposing RAW 264.7 cells to 5HT did not alter LPS-induced inflammatory cytokine response. These results suggest that the effect of paroxetine on LPS-induced

inflammatory cytokines is likely 5HT-independent. This could be explained by exploring two possible scenarios: (i) 5HT does not regulate the mechanisms of cytokine production either directly or indirectly or (ii) LPS stimulation of Mϕs could lead to increased endogenous synthesis of 5HT which desensitizes the 5HT receptor making it unresponsive to exogenous 5HT. To rule out these possibilities, in future studies 5HT levels should be measured in cells treated with LPS and paroxetine. Although, recent studies have focused on the role of 5HT and intracellular signaling [141] mediated through 5HT receptors in inflammation [118] and immune-modulation [117,127], molecular mechanisms and signaling pathways pertaining to the activity of 5HT receptors and their antagonists in inflammation continues to remain elusive.

LPS-induced macrophage activation is known to modulate various downstream inflammatory signaling pathways including NFκB, a major transcription factor regulating inflammatory cytokine production [130]. Paroxetine does not appear to influence the levels of pIκBα, pp105, pP38, pJNK1/2, and pERK1/2 (Chapter 3, Fig .3-9). These data strongly suggests that the differential regulation of cytokines could be regulated by other intracellular pathways not explored as part of this study. In summary, modulation of inflammation in Mϕs by paroxetine does not appear to act via pIκBα, pp105, pP38, pJNK1/2, and pERK1/2.

In addition to its role as effective anti-depressants, SSRIs possess anti-inflammatory activities mediated by hitherto unknown mechanisms. Identification of SSRI-mediated changes in proinflammatory cytokine secretion in Mϕs, presented in this thesis, represents a step in that direction. Thorough understanding of the molecular mechanisms and identification of potential targets in the 5HT signaling pathways might enable the use of FDA-approved SSRIs to treat conditions marked by dysregulated inflammation.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

Macrophages (Mϕs) are armed with an extensive repertoire of receptors and factors that modulate not only their function but also signal the nature of an insult to other cell types thereby shaping the course and outcome of inflammation. G-protein coupled receptor kinases (GRKs) regulate G-protein coupled receptor (GPCR)-mediated signaling in Mϕs and therefore, serve as potential drug targets to limit extensive inflammatory responses. Paroxetine, an FDA approved anti-depressant, was found to specifically inhibit GRK2. However, effect of paroxetine on GRK2 in Mϕs has not been reported and is the subject of the current work. Here, we investigated the role of paroxetine in lipopolysaccharide (LPS)-induced inflammatory cytokine response in two different types of Mϕs with an emphasis on its ability to inhibit GRK2.

Our central hypothesis was that paroxetine modulates LPS-induced inflammatory cytokine responses by inhibiting GRK2.

Specific aim 1

To elucidate the effects of paroxetine on LPS-activated primary mouse peritoneal Mϕs and RAW 264.7 cells.

Major Conclusions

- Paroxetine decreases IL-6, IL-1β but increases TNFα levels compared to the LPS-alone treated cells in a dose and time-dependant manner.

Specific aim 2

To unravel the mechanisms involved in the effect of paroxetine on LPS-induced inflammatory cytokine responses with an emphasis on its ability to inhibit GRK2 or by modulating 5HT pathways in Mϕs.

Major Conclusions

- RNA interference studies with GRK2 siRNA smartpool in RAW 264.7 cells revealed that the effects of paroxetine in Mϕs are likely GRK2-independent.
- Fluoxetine, another SSRI, revealed differential regulation of LPS-induced inflammatory cytokine response similar to that observed with paroxetine.
- Exogenous 5HT did not affect LPS-induced inflammatory cytokine response in RAW 264.7 cells pointing to a 5HT-independent role for these SSRIs.

Finally, paroxetine does not appear to influence various kinases associated with NFκB pathway including pIκBα, pp105, pP38, pJNK1/2, and pERK1/2 suggesting that differential regulation of cytokines could be mediated by other intracellular inflammatory signaling pathways.

Future Directions

Previous studies have shown that SSRIs including paroxetine possess potent anti-inflammatory properties in various experimental models of inflammation [106,110,111]. Specific GRK2 inhibitory activity of paroxetine in cardiomyocytes is not consistent with their function in Mϕs. Though our results indicate that differential regulation of LPS-induced inflammatory cytokine response by paroxetine is GRK2-independent, it could be simply due to cell-type specific effects. Also other cytokines and chemokines should be measured from similar experiments to further characterize the effects of SSRIs on inflammatory cytokine/chemokine secretion in LPS-activated macrophages. In order to further confirm these results, comparable experiments can be performed with myeloid-specific GRK2 knockout cells and different immune cell types including human monocyte/Mϕ cell lines (U937 cells, THP1 cells) to elucidate the effects of paroxetine in a broader range of immune cells derived from different species, including humans.

During various inflammatory conditions, immune and non-immune cells are activated by a variety of TLR ligands, as TLRs serve as the first line PRRs in mediating innate immunity. In this study we used only one TLR agonist, LPS, that acts as a TLR4 agonist. Anti-inflammatory roles of SSRIs should be further explored by treating the cells with ligands for other TLRs, such as peptidoglycan and flagellin. To gain insights into the activity of SSRIs in ameliorating inflammatory responses in live animal models, mono-microbial (LPS-endotoxemia) and poly-microbial sepsis (cecal-ligation and puncture model) can be utilized.

Our results suggest that paroxetine mediated effects on inflammatory cytokines is 5HT-independent. To further confirm this observation, 5HT levels in the Mϕs should be measured

before and after LPS-stimulation, which might provide answers for the unaltered cytokine response during 5HT exposure. Comparable experiments repeated with SERT knockout Mϕs would also shed light on whether paroxetine-mediated inflammatory cytokine response is 5HT-dependent.

Modulating 5HT signaling pathways by various 5HT receptor antagonists would open up new avenues for use as anti-inflammatory drugs. Understanding the molecular mechanisms pertaining to the profound inflammatory cytokine responses of SSRIs are key to take this study to the next level. This could be accomplished by screening for other intracellular inflammatory signaling pathways that could be modulated by these drugs. Completion of the proposed studies and exclusion of unintended effects of SSRIs in people without clinical depression could determine if SSRIs could be used to treat conditions caused by dysregulated inflammation.

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