Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:729-745
DOI: 10.1159/000495329
Published online: 21 November 2018
Accepted: 13 November 2018

Review

Development of Inflammatory Immune Response-Related Drugs Based on G Protein-Coupled Receptor Kinase 2

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Key Words
Inflammatory immune response (IIR) • Soft regulation of inflammatory immune responses (SRIIR) • G protein-coupled receptor kinase 2 (GRK2) • GRK2 inhibitors • Disease

Abstract
G protein-coupled receptor kinase 2 (GRK2), as a vital Ser/Thr kinase, is an important regulatory protein in the inflammatory immune response (IIR) by maintaining the balance between the function of inflammatory immune cells and non-conventional inflammatory immune cells and regulating inflammatory immune cell infiltration, inflammatory cytokine secretion, and the signaling associated with endothelial function. However, the imbalance of GRK2 expression and activity plays an important role in the development of IIR-related diseases, such as hypertension, heart failure, Alzheimer's disease, type 2 diabetes mellitus, insulin resistance, rheumatoid arthritis, thyroid cancer, multiple sclerosis, and liver cancer. Small molecule GRK2 inhibitors, including balanol, Takeda inhibitors, paroxetine and derivatives, M119 and gallein, peptides, RNA aptamers, Raf kinase inhibitory protein, and microRNAs, that can directly inhibit GRK2 kinase activity have been identified by different strategies. This review discusses recent progress in one of the hallmark molecular abnormalities of GRK2 in IIR-related diseases and explores the soft regulation of IIR by innovative drugs reducing the excessive activity of GRK2 to basal levels, without damaging normal physiological function, to ameliorate inflammatory disorders.

Introduction

The inflammatory immune response (IIR) is a physiological or excessive systemic response induced by inflammatory immune cells based on the variation of internal and external environments [1]. A physiological IIR protects the body from pathological damage caused by changes to internal and external environments. However, an excessive

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IIR eventually leads to an imbalance of the body, organs, cells, and molecules, which is the pathological basis for the generation and development of multiple systemic diseases. The mechanisms underlying an excessive IIR are still under exploration; however, T and B lymphocytes, macrophages, dendritic cells, microglial cells, endothelial cells, epithelial cells, fibroblasts, synovial cells, liver cells, inflammatory immune cytokines, and related receptor signal transduction pathways may play important roles. Thus, inhibiting an excessive IIR to restore normal physiological function is becoming a new target and strategy for treating multiple systemic diseases.

G protein-coupled receptor kinase 2 (GRK2) is a ubiquitous member of the G protein-coupled receptor (GPCR) kinase family. GRK2 contains a pleckstrin homology domain and a regulator of G protein signaling homology domain, which have multiple binding sites for several proteins and lipids such as GPCRs, G proteins (Gα and Gβγ), phospholipase C, phosphatidylinositol 4, 5-bisphosphate, extracellular signal-regulated kinase (ERK), and protein kinase A (PKA) [2]. Under normal conditions, GRK2 regulates the function of inflammatory immune cells and non-conventional inflammatory immune cells and the secretion of inflammatory immune cytokines, modulates GPCR desensitization and internalization by phosphorylation, and influences the AKT/endothelial nitric oxide synthase (eNOS) signaling pathway and nuclear factor (NF)-κB transcriptional activity to maintain normal physiological processes [3]. Under excessive IIR conditions, GRK2 expression and activation are disrupted, which triggers a change in the production and activation of eNOS, leading to endothelial dysfunction, the promotion of β-adrenergic receptor (AR) desensitization and internalization by phosphorylation, and regulation of CXCL8-stimulated CXCR2 expression, resulting in heart failure (HF)-related dysfunction, the activation of insulin-dependent negative signaling feedback, inhibition of the membrane translocation of the glucose transporter GLUT4, and increase of NF-κB transcriptional activity, which in turn leads to the production of inflammatory cytokines, the enhancement of inflammatory cell infiltration, and damage to cartilage and bone [4-6].

Considering the many proposed biological functions of GRK2, it is becoming an interesting target for the identification of drugs to regulate an excessive IIR. In drug development, it is more straightforward to screen for GRK2 inhibitors than enhancers; therefore, many studies have used this approach. This review highlights recent developments in the identification and structural analysis of GRK2 inhibitors, including balanol, Takeda inhibitors, paroxetine and derivatives, M119 and gallein, peptides, RNA aptamers, and Raf kinase inhibitory protein (RKIP). We begin by discussing what is known about the correlation between the up- or down-regulation of GRK2 and several pathological disorders to explore new targets and strategies for maintaining the dynamic balance of GRK2 expression and its activation in IIR-related diseases.

Regulation of the IIR by GRK2

An excessive IIR is induced by multiple immune cells, such as T and B lymphocytes, macrophages, dendritic cells, microglial cells, endothelial cells, epithelial cells, fibroblasts, synovial cells, and liver cells, and inflammatory immune cytokines, including interleukins (ILs), interferons (IFNs), members of the tumor necrosis factor (TNF) superfamily, chemokines, and adhesion molecules [7-16] (Table 1).

GRK2, as an important regulator of cell responses during an excessive IIR, is widely expressed in different cell types of the immune system, and regulates the signaling of chemokine receptors, including CCR5, CCR2B, and CXCR2, to modulate directed migration and the activation of T cells, monocytes, and neutrophils [17, 18]. GRK2 overexpression plays a vital role in the phosphorylation, desensitization, and internalization of GPCRs, and can increase calcium flux and ERK and AKT signaling and enhance the migration of T lymphocytes by regulating CCR5 expression in T cells of $GRK2^{+/-}$ mice [19]. Similarly, CCR2-mediated ERK1/2 signaling is increased in $GRK2^{+/-}$ splenocytes. Lipopolysaccharide-induced signaling through the Toll-like receptor 4 (TLR4) pathway down-regulates the CXCR2-induced expression of GRK2 in human polymorphonuclear neutrophils, enhancing their migration [20, 21]. GRK2 directly phosphorylates p38 at the Thr123 residue, which has an impact on p38-dependent cellular functions and alters the secretion of TNF-α upon lipopolysaccharide stimulation in $GRK2^{+/-}$ murine macrophages [22]. In addition, GRK2

can recruit PI3K to the membrane to regulate the PI3K pathway following treatment with agonists and it also interacts with AKT in endothelial cells, which results in the inhibition of AKT activity, thereby modulating the chemotactic responses of immune cells and endothelial dysfunction [23]. These observations suggest an important role for GRK2 levels in cells of the immune system. In this context, we will focus on the functional impact of the altered GRK2 levels observed in several relevant cardiovascular, nervous, endocrine, and inflammatory diseases and tumors to explore new drug targets for treating IIR-related diseases.

Table 1. Inflammatory immune response (IIR) related cells

	Derived from	Classify	Functions	References
Inflammatory immune cells				
T cells	Pluripotent stem cells of bone marrow	Naive, effector and memory T cell, Treg cells, cytotoxic T cells, Th1cells, and so on	Activation of T cells can lead to proliferation, differentiation into effector cells, and cytokine production. Tregs can induce local immune tolerance, inhibit protective anti-tumor immune responses and accumulate the tumor microenvironment correlates with reduced survival and tumor-derived.	[7, 8]
B cells	Pluripotent stem cells of bone marrow	Before B cells, immature B cells, mature B cells, activated B cells and plasma cells	Bregs have important regulatory functions on the immune response in infection, cancer and autoimmune diseases. Breg cells can produce IL-10 and inhibit the production of pro-inflammatory cytokine to suppress HIV-1 disease.	[9]
Macrophages	Blood monocytes	M1 and M2 macrophages	Macrophages participate in innate immunity and acquired immune response, and play an important role in the development of multiple diseases. M1 can release IL-1β, IL-12, IL-23, TNF-α, inflammatory chemokines, ROS and NO, and inhibit insulin signal transduction; M2 can release IL-10, TGF-β, IL-1ra and Arginase, and promote insulin sensitivity in obese mice.	[10]
Dendritic cells	Pluripotent stem cells of bone marrow.	Myeloid DC (DC1) and lymphoid DCs (DC2)	mDCs promote immune function, while iDCs show the characteristics of immune tolerance. MoDCs can increase in the IIR, lead to the development of infections and effectively against invading pathogens in the innate response.	[11]
Mast cells	Pluripotent stem cells of bone marrow	/	It can regulate vascular activity, promote MMP expression and angiogenesis. Mast cells with activated c-kit receptor are increasing in the restore blood vessels and bone marrow, which can promote stem cells growth.	[12]
Non- conventional IIR related cells				
Glial cells	Central nervous system (CNS)	Astrocytes, oligodendrocytes and microglia	Microglial cells can produce IL-1b, IL-6, TNF-α, chemokines, NO, and prostaglandins, and play critical roles in initiating and sustaining immune response to microbial, tumor, and neural antigens in the CNS.	[13]
Endothelial cells	Vascular endothelium	/	ECs play an important role in immune cell recruitment and angiogenesis, are capable of attracting immune cells to sites of inflammation or injury, and promote CD4 memory T cells activation and IFN-γ production by expressing MHC molecules, which is vital for arteriosclerosis.	[14]
Fibroblasts	Mesenchymal cells	/	CAFs can promote angiogenesis, invasion and metastasis and increase the production of pro-inflammatory cytokines, matrix degrading proteases and boost the progression and metastasis of tumor.	[15]
Synovial cells	Mononuclear macrophages of bone marrow; mesenchymal cells	MLSs, FLSs	MLSs have phagocytic function; FLSs, a significant effector cells in RA, can release PGE2, TNF-α, IL-1β, MMP-1, 2, 3, 9 to promote smooth membrane proliferation, inflammation and joint damage.	[16]

Regulatory role of GRK2 in IIR-related diseases

Cardiovascular system: Hypertension and HF

Hypertension is a multifaceted disease characterized by elevated systemic blood pressure as a consequence of increased peripheral resistance, which is the most important risk factor preceding the development of other cardiovascular diseases [24]. Low-grade inflammation has been recognized as a crucial pathogenic factor in hypertension and cardiovascular diseases [25]. Previous studies have shown that the inflammatory mechanisms of hypertension include the up-regulation of cytokine expression, such as soluble intercellular adhesion molecule-1 (ICAM-1), IL-8, monocyte chemoattractant protein-1 (MCP-1), TNF-α, IFN-γ and chemoattractant factors, and the down-regulation of nitric oxide (NO), vascular endothelial growth factor, and reactive oxygen species [26, 27].

GRK2 is overexpressed in the vascular tissue and lymphocytes of patients with hypertension (Table 2). GRK2 overexpression can inhibit AKT, shift the vascular tone toward constriction, and decrease eNOS activity, leading to endothelial dysfunction [28]. NO, as a vasodilating factor, is important in hypertension and the immune system. NO is synthesized by the endothelium to oppose the vasoconstriction induced by the sympathetic nervous system. NO has also been associated with an increase in leukocyte adhesion to mesenteric venules and an increase of monocyte/macrophage infiltration, which lead to the development of hypertension [29]. Therefore, the down-regulation of GRK2 in hypertension can increase eNOS activity and reduce high blood pressure to normal levels [30].

HF is the end stage of many underlying cardiovascular diseases, and it still has a poor prognosis despite considerable progress in pharmacological treatment and new interventional and surgical therapeutic options [31]. Previous studies have shown that HF is no longer considered as a pure cardiac entity; it involves several inflammatory pathways and immune regulation and later detrimental neurohumoral compensatory mechanisms [32]. The GPCR/TLR/IL-1 signaling pathway plays a crucial role in the regulation of cardiac function, which can result in the activation of the NF-κB system and the production of chemokines, inflammatory cytokines, and catecholamine [33]. Inflammatory cytokines, such as TNF-α, IFN-γ, IL-6, and IL-1β, can accelerate myocardial hypertrophy and fibrosis and down-modulate cardiac function during the development of HF [34]. Chemokines, including CXCL8 and CCL2, are thought to indirectly damage and cause dysfunction of cardiac muscle through the activation and production of reactive oxygen species, matrix metalloproteinases, and inflammatory cytokines.

Table 2. Summary of changes observed in GRK2 levels and function in different IIR related diseases

IIR related diseases	GRK2 expression and functions	References
Hypertension	Under physiological conditions, GRK2 can regulate Akt signaling pathway, eNOS activity and vascular functions in hypertension; GRK2 overexpression can increase eNOS activity, promote leukocyte adhesion to mesenteric venules, increase monocyte/macrophage infiltration and decrease high blood pressure which leads to the occurrence of hypertension.	[28, 29]
Heart failure (HF)	In heart, GRK2 mediates phosphorylation/desensitization of β-AR, thus inducing the HF-related dysfunction; increased GRK2 expression and activity can critically influence catecholamines secretion and is responsible for a severe adrenal β2AR dysfunction in chronic HF.	[4, 33, 34]
Alzheimer's disease (AD)	GRK2 can regulate GPCR/TLR4 signaling pathway, which play an important role in modulating immune cell function and cognitive function; In AD, GRK2 has high expression, which promotes innate immune cell migration and cardiac and vascular GPCRs desensitization and GPCRs coupling dysfunction.	[38, 39]
Type 2 diabetes mellitus (T2DM)	GRK2 involves in regulating insulin receptor and GRCR signaling and Akt/eNOS pathway, which stabilized membrane translocation of GLUT4 and endothelial function; increased GRK2 can impair the aorta of diabetic patients, and resulting endothelial dysfunction.	[43-45]
Thyroid cancer (TC)	TSH receptor belongs to GPCR, which can be regulated by GRK2 leading to activation of AC and increasing of cAMP levels, and regulation of TC cell proliferation; GRK2 overexpression can promote cell abnormal proliferation.	[49]
Rheumatic arthritis (RA)	GRK2 is associated with inflammatory cytokines production by activating NF-κB; GRK2 overexpression leads to the production of inflammatory cytokines, the enchantment of inflammatory infiltration and the damage of cartilage and bone in RA.	[55-58]

GRK2 is overexpressed in HF (Table 2). Increased cardiac GRK2 expression can promote the phosphorylation of β-AR, leading to β-AR desensitization and internalization, which play a pivotal role in inducing HF-related dysfunction [4]. Recently, it has been shown that GRK2 protein levels are markedly increased by the activation of TLR2 in macrophages. TLR2 can regulate CXCL8-stimulated CXCR2 expression by up-regulating the expression of GRK2 in circulating neutrophils [21]. Therefore, down-regulating GRK2 expression may be an important therapeutic strategy for HF by regulating the cardiac β-AR and CXCR2 signaling pathway.

Nervous system: Alzheimer's disease

Alzheimer's disease (AD) is one of the most prevalent and devastating dementing disorders, and it is characterized by the gradual loss of neurons from specific regions of the central nervous system, synaptic degeneration, accumulation of senile plaques and neurofibrillary tangles in association cortices, and an inflammatory response to amyloid-β, which can increase the adhesion of activated microglia, inflammasomes, astrocytes, and leukocytes to post-capillary venules [35]. The clinical features of AD include amnesic-type memory impairment, language deterioration, visuospatial deficits, metabolic and oxidative stress damage, and impaired cerebral perfusion. Some potential peripheral inflammatory biomarkers of AD, as neuroinflammatory triggers, can promote the activation of the peripheral immune system, including adhesion proteins, such as P-selectin, MCP-1, platelet endothelial cell adhesion molecule-1, and ICAM-1 and -2, secreted inflammatory mediators (chemokines and interleukins), and platelets contain important enzymes involved in inflammatory intermediary synthesis such as phospholipase A2 and cyclooxygenase-2 [36]. TLR4 inflammatory mediator signaling plays a pivotal role in the pathogenesis of AD, as it may be involved in AD inflammatory responses through the up-regulation of certain cytokines, such as IL-1β, IL-10, IL-17, and TNF-α [37]. GPCRs can mediate the actions of messengers of key modulators of cardiac and vascular cell function, and they are related to the neurovisceral damage and vascular complications of AD.

GRK2 is a cytosolic protein that contributes to the adaptation of GPCR/TLR4 inflammatory mediator signaling, and it regulates downstream signaling through these receptors [38]. GRK2 mRNA and protein expression are higher in patients with AD than in normal controls, and the up-regulation of GRK2 levels in peripheral lymphocytes of patients with AD includes two aspects. First, there is a moderate decrease of membrane content and a drastic increase of cytosolic content. Second, the total levels of GRK2 are also significantly up-regulated. GRK2 protein concentrations are related to the degree of cognitive impairment [39]. A previous study showed that the suppression of GRK2 translocation to the membrane can regulate TLR4 signaling-mediated monocyte migration, which may reveal a novel function for TLR4 signaling in promoting the migration of innate immune cells. GRK2 is involved in cardiac and vascular GPCR desensitization and the dysfunction of GPCR coupling in patients with AD. The data show an imbalance in the activity of NOS isoforms, ET-1, and oxidative stress, which can lead to an inadequacy in the antioxidant response capacity to abate sufficiently metabolic and oxidative insults in patients with AD. GRK2 plays a role in these deleterious processes. Thus, GRK2 might be utilized as a novel biomarker in the diagnosis and clinical monitoring of AD (Table 2).

Endocrine system: type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by insulin resistance, vascular endothelial dysfunction, and increased blood glucose levels. It can accelerate atherosclerosis and hypertension, leading to increased risks of cardiovascular morbidity and mortality [40, 41]. Macrophages and lymphocytes, as unexpected promoters and controllers of adipose tissue biology and glucose homeostasis, are important in the promotion of inflammation associated with insulin resistance and T2DM [42]. M1 macrophages can impair insulin receptor signaling in peripheral tissues by secreting TNF-α and IFN-γ, while M2 macrophages can maintain insulin sensitivity by secreting IL-10. The infiltration of B cells into adipose tissue appears to be key to the establishment of adipositis

and insulin resistance, and it can promote insulin resistance by enhancing local macrophage TNF-α production. Therefore, TNF-α is highly expressed in adipose tissue of obese animals and humans, in which it can mediate insulin resistance. Insulin suppresses hepatic glucose production and regulates glucose uptake in muscle and fat through the translocation of GLUT4 to the cell surface [43].

GRK2 is overexpressed in obese animals and humans, and a 50% down-regulation of GRK2 levels in hemizygous GRK2$^{+/-}$ mice is sufficient to protect against TNF-induced alterations in glucose homeostasis and insulin signaling, which provides strong evidence for a key role for GRK2 in the modulation of insulin sensitivity in physiological and pathological conditions [44]. GRK2 directly phosphorylates insulin receptor substrate-1, causing insulin-dependent negative signaling feedback, including inhibition of the membrane translocation of GLUT4. Previous studies have found that the activation of the AKT/eNOS pathway is impaired in the aorta of diabetic patients, resulting in endothelial dysfunction. GRK2 recognizes and phosphorylates agonist-activated GPCRs and plays a crucial role in signal transduction pathways to modulate intracellular effectors, such as endothelial function in diabetic mice, which exerts this effect by ameliorating AKT/eNOS dysfunction [45] (Table 2).

Cancer: thyroid carcinoma

Thyroid carcinoma (TC) is the most common endocrine malignant tumor, which originates from both follicular and parafollicular cells. TC is classified as a well-differentiated carcinoma, including papillary carcinoma and follicular carcinoma, and an undifferentiated or anaplastic carcinoma [46]. Lymphocytic infiltration results from the local recurrent activation and proliferation of circulating leukocytes and plays a key role in the initiation and development of TC [47]. Cells migrate from the circulation into tissues following the release of cytokines, such as TNF-α, IL-lβ, and IFN-γ, and up-regulate the expression of adhesion molecules, such as ICAM-1, vascular cell adhesion molecule 1, and E-selectin, on endothelial cells. The up-regulation of ICAM-1 expression may play an important role in thyroid tumors and they may interact with the immune system and have a cytotoxic effect [48].

The thyroid stimulating hormone (TSH) receptor is a seven-transmembrane domain receptor belonging to the GPCR family. The effect of TSH on GRK2 expression was mimicked by forskolin, which can activate adenylyl cyclase (AC) and increase cAMP levels. Studies have shown that compared with their adjacent normal tissues, GRK2 expression is increased in TC, including papillary, follicular, and anaplastic types, because TSH, insulin, and insulin-like growth factor-I are involved in the progression of TC. GRK2 overexpression can promote the proliferation of TC cell lines. The reduction of cell proliferation by GRK2 revealed a new role for this kinase in the growth of TC. The mechanism may involve the reduction of lymphocytic infiltration and the down-regulation of cytokines and adhesion molecules, such as TNF-α, IL-lβ, IFN-γ, and ICAM-1[49] (Table 2).

Immune system disease: rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by the inflammation of joint tissue and synovial hyperplasia with the proliferation of fibroblast-like synoviocytes (FLS), which eventually leads to cartilage and bone destruction. The synovial environment in RA is comprised of a complex mix of cell types including T and B cells, neutrophils, monocytes/macrophages, and FLS. It has been shown that the activation of autoreactive T and B cells stimulates macrophages to produce pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [50, 51]. FLS, as synovial fibroblasts or type B synoviocytes, are the predominant cell type comprising the structure of the synovial intima and the most common cell type in the pannus-cartilage junction, and contribute to joint destruction through their production of cytokines, chemokines, and matrix-degrading molecules by migrating to and invading joint cartilage in RA [52]. Prostaglandin E2, an important inflammatory cytokine, is increased in FLS. It regulates a broad range of physiological processes in the immune system as well as the cardiovascular, endocrine, and neural systems. It also produces a response to pro-inflammatory cytokines, which in turn negatively regulates IL-17 and TNF-α expression and the TNF/IL-1-induced activation of FLS through EP2/EP4 receptors, and results in the modulation of pro-inflammatory cascades. The NF-κB pathway, one of the major signaling pathways in FLS, plays a pivotal role in joint inflammation. NF-κB is highly activated in the synovial tissue of patients with RA and in collagen-induced arthritis (CIA) mice [53, 54]. NF-κB regulates the expression of various pro-inflammatory mediators,

including cytokines, such as TNF-α, IL-1β, and IL-6, chemokines, and cellular adhesion molecules. NF-κB activation in FLS also contributes to the pathogenesis of RA by activating the transcription of matrix metalloproteinases, which are responsible for the invasive properties of FLS [55, 56]. GRK2 expression is increased in the synovium of CIA rats during inflammation [57]. GRK2 overexpression can regulate the EP receptor, leading to EP receptor desensitization, a decrease of cAMP levels, and the proliferation of FLS (unpublished). GRK2 is associated with an increase of NF-κB transcriptional activity and up-regulation of NF-κB activity, leading to the production of inflammatory cytokines, enhancement of inflammatory cell infiltration, and damage to cartilage and bone [58] (Table 2).

Development of GRK2 inhibitors

GRK2, which is 689 amino acids (aa) in length, has three domains: an N-terminal domain, kinase domain, and C-terminal domain. The structural features of GRK2 have played a vital role in the development of GRK2 inhibitors. Its C-terminal domain contains approximately 54 aa (618–671 aa) and has three important regions: the NLT includes the HF motif and interacts with the β-helix, C-helix, and β4 strand of the N lobe; the active-site tether (AST) serves as a gate for ATP and substrate binding and peptide substrate recruitment; and the CLT interacts with the E-helix of the C lobe and with the αC-β4 loop, β8 strand, and the interlobe linker spans the N and C lobes [59]. The kinase domain of GRK2 (185–513 aa), as an ATP binding site, contains various α-helixes and β-turns (Fig. 1). The Gβγ binding sites are located at 1–53 aa and 643–670 aa; the Gαq binding sites are located at 20–185 aa. GRK2 also contain small lobes (186–272 aa and 496–513 aa), hinge region (273–275 aa), large lobe (276–495 aa), P-loop (191–201 aa), activation loop (containing Ser334), and αB-αC-helix (224–248 aa), which form important binding sites for GRK2 inhibitors to regions that are GRK2 "allosteric sites" [60].

GRK2, as a relevant modulator of inflammatory responses, can interact with various signal transduction proteins related to the chemokine-induced migration of T cells and monocytes, such as MEK, AKT, PI3Kγ, and GIT. GRK2 expression and function is altered in several inflammatory conditions and has emerged as an important protein in the onset or progression of IIR-related diseases. Therefore, GRK2 is a new drug target for the treatment of some diseases. At present, GRK2 inhibitors include balanol, Takeda inhibitors, paroxetine and derivatives, M119 and gallein, peptides, RNA aptamers, RKIP, and microRNAs (miRNAs), which have different structures, inhibition effects, and inhibition mechanisms (Table 3).

Small molecule GRK2 inhibitors

Balanol

Balanol, as an ATP mimetic, is a metabolite synthetized by the fungus *Verticillium balanoides*, and it functions as a competitive inhibitor of ATP at the kinase domain of GRK2 [61]. The X-ray crystal structures of balanol and balanol analogs bound to PKA revealed that the compound binds to a conformation of PKA, which forms an intermediate state to the "open" (inactive) and "closed" (active) states of the kinase. Balanol exhibits some selectivity among GRK subfamilies, and it seems to recognize and stabilize a unique inactive conformation of GRK2. Cocrystallization of balanol-GRK2 complexes indicated that balanol binding to GRK2 can lead to conformational changes of GRK2 in the P-loop and in the αB-αC loop of the small lobe. Analysis of the binding sites of balanol and GRK2 showed that Gly201, Val205, Lys220, Glu239, Met274, Asp272, Ala321, Leu324, and Asp335 of GRK2 may bind to balanol by hydrogen bonding (Table 4A).

Paroxetine

Paroxetine is a selective serotonin reuptake inhibitor anti-depressant drug that can specifically bind to the kinase domain of GRK2 as an off-target with a binding affinity in the micromolar range and selectively inhibits GRK2 kinase activity [62]. Paroxetine stabilizes the kinase domain of GRK2 in a conformation that could serve as a unique platform for the development of GRK2-specific inhibitors, which is distinct from the other classes of currently known GRK2 inhibitors as well as from other protein kinase A, G, and C family members.

Table 3. The development of GRK2 inhibitors

	PDB-ID	IC50 (nM)	The mechanism of inhibition	Development and efficacy of inhibitors	References
GRK2●inhibitor complexes					
GRK2●balanol	3KRW	42	Recognize and stabilize a unique inactive conformation of GRK2	Not be used to animal model or cell experiments.	[61]
GRK2●paroxetine	3V5W	5000	It can specifically bound to the catalytic domain of GRK2 as an off-target and inhibited kinase activity	Paroxetine considerably improved left ventricular function and structure in a mouse model for MI; it also markedly improves LVEF, fractional shortening, and left ventricular dysfunction in AMID patients.	[62-64]
GRK2●GSK180736A	4PNK	2500	The inhibitor of GRK2 co-crystallized in the active site.	Not be used to animal model or cell experiments.	[65]
GRK2●Takeda101	3PVU	290	P-loop and αB-αC-loop region could contribute to the selectivity of these compounds for GRK2	Not be used to animal model or cell experiments.	[66]
GRK2●Takeda103A	3PVW	54	P-loop and αB-αC-loop region could contribute to the selectivity of these compounds for GRK2	Not be used to animal model or cell experiments.	[66]
M119 and gallein	/	/	It can inhibit Gβγ-GRK2 interactions	M119 and gallein partially normalized cardiac morphology and gene expression and halted HF progression in the acute Iso pump model of HF and in CSQ mice with established HF.	[67]
Other GRK2 inhibitors					
RNA aptamers		4~220	Such as C13 , it stabilizes a unique inactive conformation of GRK2	Not be used to animal model or cell experiments.	[68, 69]
microRNA	/	/	miR-K3 directly targeted GRK2	miR-K3 promoted endothelial cell migration and invasion by directly targeting GRK2.	[70]
PEPTIDES GRK2ct (βARKct)	/	/	It can translocate the plasma membrane and bind to Gβγ	GRK2ct can restore cardiac function in mouse models with heart failure.	[71]
PEPTIDES 383-390 KLLRGH SP of GRK2	/	47,000~ 55,000	It can selectively inhibit GRK2	Not be used to animal model or cell experiments.	[72, 73]
PEPTIDES GRK2-specific inhibitor peptide (GRKInh)	/	>40,000	It can selectively inhibit GRK2	GRKInh counteracts the heart failure-related cardiac metabolic dysfunction and signs of heart failure of FASN transgenic mice.	[74]
Raf kinase inhibitory protein (RKIP)	/	/	Phosphorylation by PKC also switches RKIP's association from Raf-1 to GRK2, leading to inhibition of GRK2 activity	In cardiomyocytes, the downregulation of RKIP inhibits beta-adrenergic signaling and contractile activity.	[75, 76]

Structural analysis of the GRK2-paroxetine complex indicated that the drug occupies the P-loop (191–201 aa) and the AST (474–492 aa) [63, 64]. Paroxetine may bind to GRK2 by hydrogen bonding with Arg199, Gly200, Val205, Asp278, and Leu324, and the complex forms specific hydrogen bonds to the backbone atoms of Asp272, Met274, and Ala321 (Table 4B).

Derivatives of paroxetine

GSK180736A, a derivative of paroxetine, is structurally similar to paroxetine and was developed as a Rho-associated coiled-coil kinase 1 inhibitor. Cocrystal structures of GSK180736A and GRK2 showed that GSK180736A co-crystallized in the active site, and it

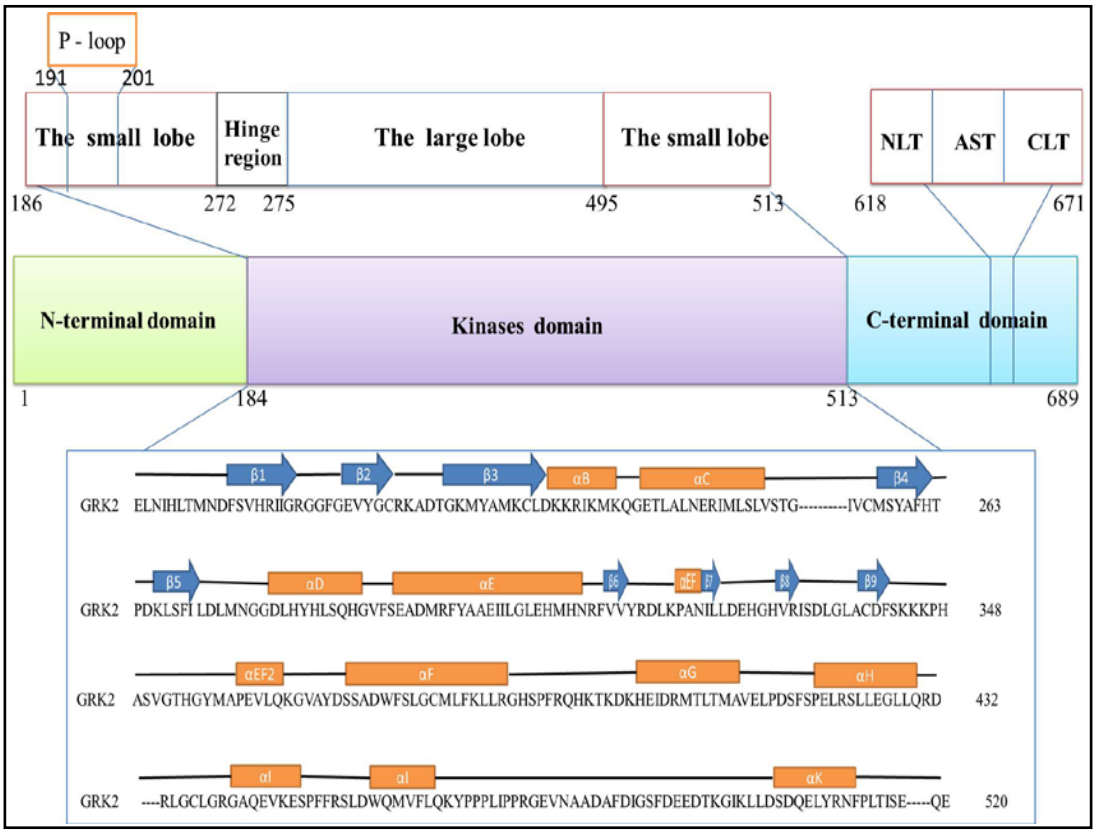


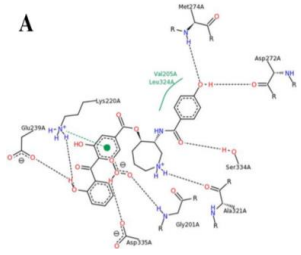
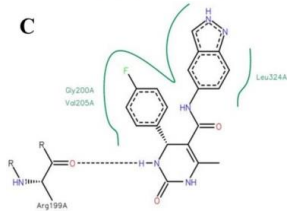
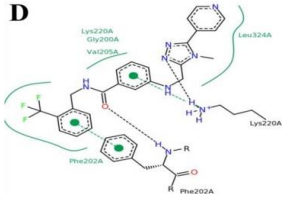
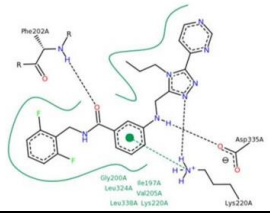
Fig. 1. The structural and functional feature of GRK2 domain GRK2 contains N-terminal region (green), kinases domain (purple) and C-terminal domain (blue). The KD of GRK2 with ~ 328aa has some important α-helix and β-turn. It contains the small lobe (186-272aa, 496-513aa), hinge region (273-275aa), the large lobe (276-495aa), the P-loop (191-201aa), the activation loop (contains Ser334), αB-αC-helix (224-248aa), which form the important GRK2 inhibitors binding to regions that GRK2 "allosteric site". The C-terminal domain of GRK2 with ~ 54aa has the N-lobe tether (NLT), the active-site tether (AST), the C-lobe tether (CLT).

was identified as an inhibitor of GRK2 [65]. Analysis of the binding sites of GSK180736A and GRK2 showed that Arg199, Gly200, Val205, and Leu324 of GRK2 may bind to GSK180736A by hydrogen bonding, and Arg199 in the P-loop and van der Waals interactions with residues in the large lobe; the compound makes specific hydrogen bonds to the backbone atoms of Asp272, Met274, and Ala321 (Table 4C).

Takeda inhibitors

Takeda inhibitors (CMPD101 and CMPD103A), as heterocyclic compounds, exhibit higher selectivity and show more therapeutic potential for GRK2 than does balanol. They were discovered by the Takeda Pharmaceutical Company Ltd. CMPD101 and CMPD103A inhibit GRK2 by inducing a slight closure of the kinase domain of GRK2, because they bind to GRK2 in an open, non-catalytic conformation [66]. Crystal structures of Takeda inhibitor-bound GRK2 showed that they bind to the active sites of GRK2 including the large lobe, P-loop, and αB-αC-loop, and GRK2 subfamily-specific residues in the P-loop and αB-αC-loop regions could contribute to the selectivity of these compounds for GRK2. Gly200, Phe202, Val205, Lys220, and Leu324 of GRK2 may form hydrogen bonds with CMPD101 (Table 4D), and Ile197. Gly200, Phe202, Val205, Lys220, Leu324, Asp335, and Leu338 of GRK2 may form hydrogen bonds with CMPD103A (Table 4E).

Table 4. Poseview image and the hydrogen bond of GRK2•inhibitors

Poseview image of GRK2•inhibitor	Structural analysis of GRK2 inhibitors	Binding in the active site of GRK2	Hydrogen bond of GRK2•inhibitor	References
A 	Balanol has four rings with hydroxybenzamide, azepane and benzophenone.	The P-loop and the αB-αC loop of the small lobe.	Gly201, Val205, Lys220, Glu239, Met274, Asp272, Ala321, Leu324, Asp335 of GRK2	[61]
B 	Paroxetine is benzolactam derivative with fluorophenyl and a benzodloxole ring.	The P-loop, the active-site tether (AST) and hinge region	Arg199, Gly200, Val205, Asp278, Leu324, Asp272, Met274, and Ala321 of GRK2	[62-64]
C 	GSK180736A has fluorophenyl, dihydropyrimidine ring and indazole. The role of indazole as the benzodioxole ring of paroxetine.	The P-loop, the large lobe and hinge region	Arg199, Gly200, Val205, Leu324, Asp272, Met274, and Ala321 of GRK2	[65]
D 	Takeda inhibitors (CMPD101) has four rings with pyridine, 1,2,4-triazole, aminobenzamide, substituted benzene.	The large lobe, the P-loop, and the αB-αC-loop	Gly200, Phe202, Val205, Lys220, Leu324 of GRK2	[66]
E 	Takeda inhibitors (CMPD103A) has four rings with pyrimidine, 1,2,4-triazole, aminobenzamide, substituted benzene;	The large lobe, the P-loop, and the αB-αC-loop	Ile197, Gly200, Phe202, Val205, Lys220, Leu324, Asp335, Leu338 of GRK2	[66]

M119 and galleon

M119 and its highly homologous structure, gallein, demonstrated the ability to reduce GRK2 recruitment to the membrane of cardiomyocytes induced by iso treatment by enhancing β-AR signaling *in vitro*, and have a high apparent affinity for Gβγ [67]. Thus, they were used to inhibit Gβγ-GRK2 interactions and selected as lead drugs to define structure-activity requirements for binding to Gβγ. M119 and gallein can both terminate the progression of HF and improve cardiac function, morphometry, histology, and gene expression in animal models of either new onset or established HF.

Other GRK2 inhibitors

RNA aptamers. C13, as an RNA aptamer, can selectively inhibit GRK2 with high affinity. It can stabilize a unique inactive conformation of GRK2 through multiple interactions leading to the inhibition of GRK2 [68]. Both within and outside the kinase domain active site pocket, the terminal stem of the aptamer indirectly contributes to its selectivity by constraining a selected portion of the RNA. The RNA sequence of C13.18 is CCAUACGGGAGAGAAACU, and it

inhibits GRK2 at the nanomolar level (IC50 = 220 ± 40 nM). Studies indicated that the C13.18 aptamer binds to the αF-αG loop and αG helix of GRK2 (391–410 aa; PDB entry 2BCJ) [69].

Kaposi's sarcoma-associated herpes virus (KSHV) is linked with Kaposi's sarcoma and lymphomas and encodes 12 precursor miRNAs within its latency-associated region, which are processed into at least 25 mature miRNAs named KSHV-miR-K12-1~12 (or simply as miR-K1~12). Of the 12 viral KSHV-encoded miRNAs that function as regulators by maintaining viral latency and inhibiting viral lytic replication, the expression of miR-K3 in latent KSHV-infected cells can suppress both viral lytic replication and gene expression. miR-K3-targeted GRK2 can promote the migration and invasion of endothelial cells by inducing CXCR2 expression and activating AKT signaling, which was initially identified as a Ser/Thr kinase implicated in the regulation of GPCRs with arrestins and the modulation of cell motility by the complex [70].

Peptides. GRK2ct (β-ARKct) peptide, as a GRK2 inhibitor, was first designed based on the knowledge that the main function of the GRK2ct peptide, the C-terminal fragment of GRK2, is to inhibit endogenous GRK2 by translocating to the plasma membrane and binding Gβγ. GRK2ct peptide is approximately 200 aa in length [71].

The catalytic fragment (383–390 KLLRGHSP) of GRK2 is composed by the last part of the α-helix F (residues 383–386) and the first part of the α strand (residues 387–390) within the HJ loop. Several crystallographic and mutational studies have reported that HJ-αG residues are involved in substrate binding and in binding to upstream activators [72, 73]. A GRK2-specific peptide inhibitor (GRK-Inh) was derived from the first intracellular loop of hamster β2-AR and has a peptide sequence of MAKFERLQTVTNYFITSE. Studies have confirmed the cardioprotective effect of GRK-Inh by increasing the activation of the MAPK signaling pathway, which oversees the processes of cell growth and proliferation. Thus, treatment with GRK-Inh can prevent cardiomyocyte apoptosis and the consequent impairment of heart function [74].

RKIP. RKIP, as a multifaceted kinase modulator, belongs to an evolutionarily conserved family of phosphatidylethanolamine-binding proteins. It can control vital intracellular signaling pathways, including the signaling cascades of Raf/MEK/ERK, NF-κB, glycogen synthase kinase-3, and GPCRs. Raf-1 controls the proliferation and differentiation of different cell types by phosphorylating and activating ERK1 [75]. The enhanced dimerization of RKIP translates into decreased Raf-1 levels and increased GRK2 inhibition. Ach can induce RKIP phosphorylation, promote the association of RKIP and GRK2, decrease the association of RKIP and Raf-1, and stimulate ERK1/2 activity. Phosphorylation by PKC also switches the association of RKIP from Raf-1 to GRK2, leading to the inhibition of GRK2 activity [76].

Conclusion

GRK2 is a regulator of immune cell function, inflammatory factor secretion, and related signaling pathways. Studies have shown that GRK2 is highly expressed in different types of immune cells, and it can attenuate the chemokine-induced migration of T cells and monocytes as a relevant modulator of inflammation [77]. GRK2 phosphorylates chemotactic GPCRs, such as CCR5, CCR2b, CXCR4, and CXCR2, and chemotactic receptors for substance P, S1P or formyl-peptide, and can also modify PDGF or EGF plasma membrane tyrosine kinase receptors, cytoskeleton proteins (ezrin and tubulin), p38MAPK, and metabotropic glutamate receptors, which contribute to leukocyte trafficking to inflammatory foci, T cell egression from lymphoid organs, and leukocyte activation or proliferation [78]. GRK2 can reduce lymphocytic infiltrates, and down-regulate the production of cytokines and adhesion molecules, such as TNF-α, IL-lβ, IFN-γ, and ICAM-1. NF-κB regulates the expression of various pro-inflammatory mediators, including cytokines, such as TNF-α, IL-1β, and IL-6, chemokines, and cellular adhesion molecules. GRK2 expression is increased in the synovium,

which can activate NF-κB in FLS, leading to the production of inflammatory cytokines. Thus, GRK2 is up-regulated in IIR-related diseases, including hypertension, HF, AD, T2DM, RA, and TC.

Interestingly, GRK2 protein expression and kinase activity are down-regulated in leukocytes of patients with active relapsing-remitting multiple sclerosis (MS) or with secondary progressive MS [79]. MS is a chronic inflammatory disease of the central nervous system, and the pathogenesis of MS involves the infiltration of activated T lymphocytes and macrophages into the brain and spinal cord. Previous data demonstrated that a 50% decrease of GRK2 expression in T cells from $GRK2^{+/-}$ mice results in an increased chemotactic response to the chemokines CCL3, CCL4, and CCL5 via the CCR5-mediated signaling pathway [19]. Reduced cellular levels of GRK2 can increase not only the response to chemokines but also to multiple GPCRs, including β-AR. These chemokines can also increase ERK, protein kinase B, and calcium levels. Studies have demonstrated that GRK2 overexpression has an inhibitory role in the growth of hepatoma carcinoma cells [80]. Dominant-negative GRK2-K220R or a GRK2-specific peptide inhibitor increases tumor mass. The activity of the MAPK signaling pathway can promote tumor growth, while GRK2 overexpression can suppress MAPK activation and nuclear translocation, GRK2 inhibitors enhance the MAPK pathway, induce nuclear ERK1/2 targets, notably the proto-oncogene FOS, and promote tumor growth [81].

These data clearly implicate the functional importance of "normal" GRK2 levels for the body (Fig. 2). The dynamic equilibrium of GRK2 expression and activity also plays an important role in the development of IIR-related drugs. The development of IIR-related innovative drugs targeting GRK2 should pay attention to controlling the excessive activity of GRK2 to the basal level, and not damage normal physiological function, and to ameliorate inflammatory disorders. Our group first put forward and clearly defined the new concept of "soft regulation of inflammatory immune responses (SRIIR)", and SRIIR, as a new field of IIR-related innovative drug development, places an emphasis on controlling the excessive activity of IIR-related cells and not to impair normal physiological function. At present, the conventional IIR drugs in the clinical setting, such as non-steroidal anti-inflammatory drugs, steroidal anti-inflammatory drugs (glucocorticoid hormones), immunosuppressive medicine (chemical medicine, biological medicine, and Chinese natural medicine) in the treatment of IIR-related diseases, have a good curative effect in treating IIR-related diseases, but result in serious adverse reactions in the liver, kidney, gastrointestinal tract, respiratory, blood, and cardiovascular systems, bone marrow, and nervous system. SRIIR drugs do not completely suppress the functions of cells or gene and protein expression and activity, but selectively control excessive activity to basal levels to restore the dynamic balance of cells, which can better exert the treatment effects of drugs and minimize the occurrence of adverse reactions. GRK2 may regulate IIR by reducing the infiltration of inflammatory cells and the release of inflammatory cytokines, which play a vital role in treating IIR-related diseases. Thus, GRK2, as a definitive example for the development of SRIIR-related innovative drugs, is becoming a new target for the treatment of IIR-related diseases.

Abbreviations

Tregs (CD4+CD25+foxp3+ regulatory T cells); Th1cells (CD4+ T helper 1 cells); Bregs (regulatory B cells); IL-1ra (IL-1 receptor antagonist); mDCs (mature dendritic cells); iDCs (immature dendritic cells); MoDCs (monocyte-derived dendritic cells); CAFs (Cancer-associated fibroblasts); MLSs (macrophage-like synoviocytes); FLSs (fibroblast-like synoviocytes).

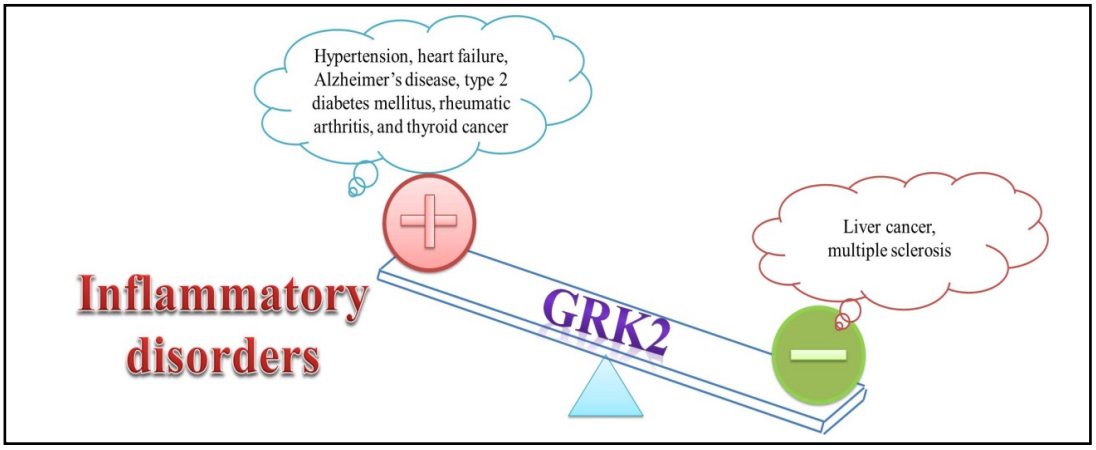


Fig. 2. The expression of GRK2 is association with the development of IIR related diseases.

Acknowledgements

This research was supported by National Natural Science Foundation of China (No. 81330081; 81673444; 81502123).

Disclosure Statement

The authors have no conflicts of interest.

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