AMERICAN UNIVERSITY OF BEIRUT

IMPACT OF KALLIKREIN KININ SYSTEM COMPONENTS ON MACROPHAGES' INFLAMMATORY RESPONSE UNDER NORMAL AND HYPERGLYCEMIC CONDITIONS

by
LAMA MOHAMAD SABER

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LAMA MOHAMAD SABER

Approved by:

Dr. Ayad Jaffa, Professor and Chair
Biochemistry and Molecular Genetics — Advisor

Dr. Aida Habib Abdul-Karim, Professor
Biochemistry and Molecular Genetics — Member of Committee

for Dr. Firas Kobeissy, Associate Professor
Biochemistry and Molecular Genetics — Member of Committee

Dr. Miran Jaffa, Associate Professor
Epidemiology and Population Health — Member of Committee

Date of thesis defense: September 8, 2020

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AN ABSTRACT OF THE THESIS OF

Lama Mohamad Saber for Master of Science

Major: Biochemistry

Title: Impact of Kallikrein Kinin System Components on Macrophages' Inflammatory Response under Normal and Hyperglycemic Conditions

Background: Inflammation is greatly associated with the development of chronic diseases such as diabetes. Diabetes is the leading cause of death in the world since it can lead to many macrovascular and microvascular (nephropathy, retinopathy, and neuropathy) complications. The Kallikrein-kinin system (KKS) contributes to inflammation through generation of bradykinin and activation of bradykinin receptors and protease activated receptors (PARs). Components of the KKS has recently been implicated as a modulator of diabetic microvascular and macrovascular complications. Higher plasma kallikrein (PK) activity is associated with higher blood pressure and increased albumin excretion rate in type 1 diabetic subjects. Furthermore, circulating levels of plasma PK are associated with carotid intima-media thickness and its progression in subjects with type 1 diabetes. Collectively, these data suggest that KKS components may play a potential role as a risk marker of inflammation-induced tissue injury.

Aims: In the present study we aimed to determine whether hyperglycemia will modulate the expression of KKS components in macrophages. Also, to detect the effect of the KKS components on macrophages inflammatory response under normal and high glucose concentrations.

Methods: THP-1 macrophages and mice bone marrow derived macrophages (BMDM) were exposed to normal glucose (5.5mM) and high glucose concentration (20mM) for 24 hours to assess their effects on the expression of KKS components and polarization of macrophages. In the second series of experiments we assessed the effects of KKS components on inflammation and polarization of macrophages under normal and hyperglycemic conditions. Gene expression profiles (bradykinin receptors, PARs, cytokines, and fibrotic factors) were measured by RT- qPCR and release of inflammatory cytokine (IL-6) was measured by ELISA.

Results: Our preliminary results indicated that under normal glucose levels B2R, KNG-1 and TBXA2R genes expression were induced when THP-1 macrophages were stimulated with bradykinin (BK) and plasma kallikrein (PK). However, high glucose levels increased the expression of PAR2, PAR1 and B2R when stimulated with PK and BK. Moreover, kininogen and B1R levels elevated in response to BK under high glucose conditions. In addition, it was observed that high glucose promoted the release of interleukin 6 after

stimulation of THP-1 with BK and PK. For bone marrow derived macrophages high glucose and stimulation with PK and BK showed an increase in B1R, B2R and PAR1 gene expression.

Conclusion: These studies indicate that high glucose levels modulate the expression of KKS components in macrophages. Furthermore, these data demonstrate that components of the KKS can promote macrophages' inflammatory response.

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ABBREVATIONS

ARG-1	Arginase-1
ADA	American diabetes association
ACE	Angiotensin converting enzyme
B1R	Bradykinin receptor 1
B2R	Bradykinin receptor 2
BK	Bradykinin
BMDM	Bone marrow-derived macrophages
CNS	Central nervous system
CVD	Cardiovascular disease
Ca^{2+}	Calcium
cGMP	Cyclic guanosine monophosphate
cAMP	Cyclic adenosine monophosphate
CTGF	Connective tissue growth factor
Cox-2	Cyclooxygenase-2
DAMPs	Damage-associated molecular patterns
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
eNOS	Endothelial nitric oxide synthase
EGFR	Endothelial growth factor receptor
ELISA	Enzyme-linked immunosorbent assay

FXII	Factor 12
FXIIa	Active factor 12
FXI	Factor 1
FXIa	Active factor 1
FBS	Fetal bovine serum
F2RL1	Coagulation factor II (thrombin) receptor-like 1
F2R	Coagulation factor II thrombin receptor
GPCR	G protein coupled receptor
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Ga1-3	Galectin-3
HMWK	High molecular weight kininogen
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IL-1β	Interlukin-1 beta
IL-6	Interlukin-6
IL-12	Interlukin-12
IL-23	Interlukin-23
IL-4	Interlukin-4
IL-13	Interlukin-13
IL-10	Interlukin-10

IFN-γ	Interferon gamma
IP3	Inositol triphosphate
JAK/STAT	Janus kinase/Signal transducer and activator of transcription
KKS	Kallikrein kinin system
KNG-1	Kininogen-1
LPS	Lipopolysaccharide
LDL	Low density lipoprotein
LMWK	Low molecular weight kininogen
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
NFκB	Nuclear factor of light chain enhancer of B cells
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PRRs	Pathogen recognition receptors
PAD	Peripheral artery disease
PLCβ	Phospholipase Cβ
PIP2	Phosphatidylinositol biphosphate
PG	Prostaglandin
PLA	Phospholipase A2
PKC	Protein kinase C
PARs	Proteinase activated receptors
PI3K	Phosphoinositide 3 kinase

PK	Plasma kallikrein
P/S	Penicillin/Streptomycin
RBL	Red blood cell lysis
RT-PCR	Real-time polymerase chain reaction
RPMI	Roswell Park Memorial Institute
Th1	T helper type 1
TNF-α	Tumor necrosis factor-alpha
Th2	T helper type 2
TGF-β	Transforming growth factor beta
TLRs	Toll-like receptor
TBXA2R	Thromboxane A2 receptor
TMB	3,3',5,5'-tetramethylbenzidine
VSMCs	Vascular smooth muscle cells

CHAPTER I

INTRODUCTION

A. Inflammation:

1. Inflammation Overview:

Inflammation is a protective process evolved in response to tissue injury, microbial stimuli, pathogens, and other detrimental insults. It is an important immune response that eliminates harmful stimuli and ensure healing of affected tissue (Ahmed, 2011). At tissue level, inflammation induces some feature characteristics such as redness, swelling, heat, pain, and loss of function (Chen et al., 2018). Innate immune cells (neutrophil, macrophages, mast cells, dendritic cells, and lymphocytes) play a major role in the initiation of inflammation (Akira et al., 2006). The mechanism of inflammation is an organized and dynamic process (Abdulkhaleq et al., 2018). It starts with recognition of pattern-associated-molecular-patterns (PAMPs) and damaged associated molecular patterns (DAMP) by pattern recognition receptor (PRR) found on immune and non-immune cells. After recognition, many inflammatory pathways are triggered such as NF-KB, MAPK, and JAK-STAT pathways. These inflammatory pathways can affect the pathogenesis of many chronic diseases and induce the production of inflammatory mediators. After that, immune cells are recruited to the site of injury due to the release of chemokines, cytokines, and growth factors. The first immune cells to be recruited are neutrophils followed by monocytes, lymphocytes, T and B cells, and the mast cells (Chen et al., 2018). At the end of the inflammatory process, it is important to prevent the progression of acute

inflammation into chronic inflammation. Resolution of inflammation is accomplished by reduction of neutrophil recruitment, transition of pro-inflammatory macrophages into anti-inflammatory macrophages, and regulation of chemokines and cytokines release leading to healing of damaged tissue. However, if acute inflammation was not resolved this will lead to persistent, chronic inflammation which can result in a host of diseases such as diabetes, cardiovascular disease, atherosclerosis, arthritis, and cancer (Nathan & Ding, 2010).

2. Macrophages:

a. Origin and Tissue Distribution:

Monocytes are small population of white blood cells that originate in the bone marrow from myeloid progenitor cells. These myeloid progenitor cells are known as granulocyte/macrophage colony forming unit they first give rise to monoblasts then promonocyte and finally monocytes. Monocytes migrate from the bone marrow into peripheral blood then into various tissues where they differentiate into specialized tissue macrophages (Fig.1). Tissue specific macrophages have been given variety of names based on their different properties, phenotypes, and anatomical location. Thus, kupffer cells are found in liver, microglial cells in the CNS, mesangial cells in the kidney, osteoclast in the bones, histocytes in spleen and connective tissues, as well as number of other macrophages types. Although, macrophages are known to possess tissue-specific functions, all tissue resident macrophages mainly have phagocytic properties they engulf debris, microbes, and foreign antigens. (Mosser & Edwards, 2008)

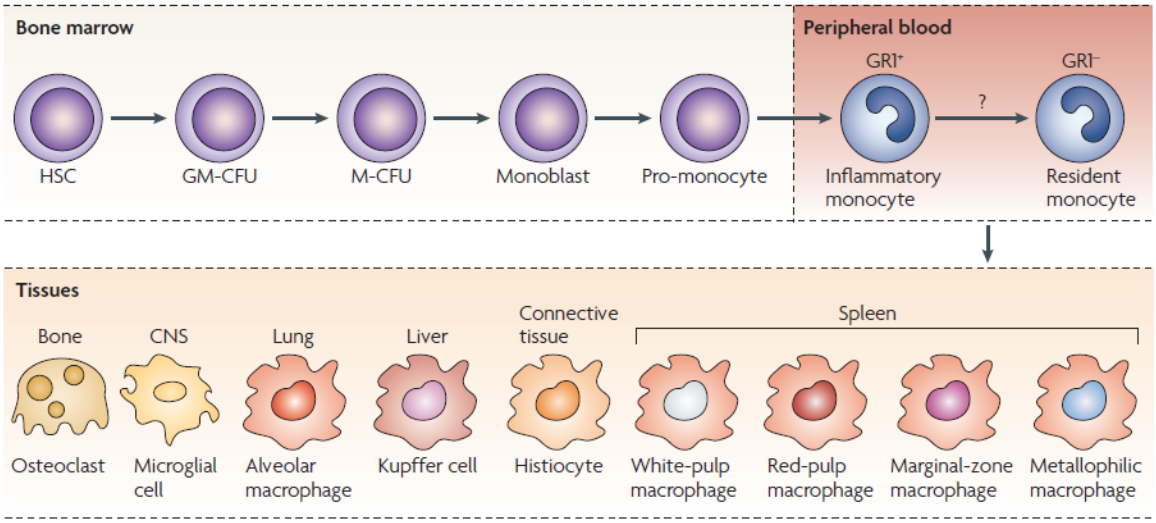

Figure 1: The Origin of Macrophages and its Differentiation in Various Tissues (Mosser & Edwards, 2008)

b. Macrophage Polarization:

Macrophages can switch from one phenotype to another based on environmental stimuli and signals that can arise in specific tissue. This phenomenon is known as macrophage polarization. There are several classes of macrophages that were identified in humans and mice depending on their cell surface markers, function, and cytokines production. Hence, macrophages are classified in to two major subtypes which are classically activated or inflammatory macrophages (M1) and alternatively activated or anti-inflammatory macrophages (M2) (Meshkani & Vakili, 2016).

M1 phenotype usually activated by Th1 cytokines such as IFN-γ and TNF-α, or by bacterial stimuli as LPS. This activation triggers the release of pro-inflammatory cytokines which are IL-1β, TNF, IL-6, IL-12, IL-23. Moreover, M1 macrophages are characterized by their antimicrobial properties, antitumor activity, and ability to secure resistance against pathogens and infections. However, their activation needs to be tightly controlled since the

cytokines and mediators they produce can lead to tissue damage, initiation and sustaining of inflammation which can be detrimental to health. (Mosser & Edwards, 2008)

In contrast, M2 phenotype is induced by fungal cells, helminth infection, apoptotic cells, and Th2 cytokines which are IL-4 and IL-13. So M2 polarization leads to high production of IL-10, TGF-β, and Arg-1. Functionally, M2 macrophages possess potent phagocytic capacity, wound healing, tissue remodeling, tumor formation, and dampening of inflammation. (Shapouri-Moghaddam et al., 2018)

B. Diabetes:

1. Overview of Diabetes:

Diabetes is a metabolic disease characterized by high fasting blood glucose caused by deficiency in insulin action, insulin secretion or both (Kharroubi, 2015). According to the American Diabetes Association (ADA) 347 million people suffer from diabetes worldwide (Sajid, n.d.). Diabetes can present with several symptoms such as polyuria, polyphagia, polydipsia, blurred vision, and weight loss (*Diabetes WHO.Pdf*, n.d.). Furthermore, chronic hyperglycemia associated with diabetes can lead to end organ dysfunction and failure including kidney, nerves, heart, blood vessels, and retina. Diabetic complications are the leading cause of morbidity and mortality in the world. They are divided in to two categories microvascular and macrovascular. Microvascular complications involve retinopathy, neuropathy, and nephropathy; on the other hand, macrovascular complications include cardiovascular disease, stroke, and periphery artery

disease (PAD) (Fowler, 2011). Diabetic foot ulcer is one of the complications that is defined as foot ulcer accompanied with PAD, neuropathy, and infection; moreover, it is the main cause of lower limb amputation (Papatheodorou et al., 2018).

2. Classifications:

ADA classified diabetes in to 3 types which are type 1 diabetes (insulin dependent diabetes), type 2 diabetes (insulin independent diabetes) (Fig.2), and gestational diabetes (Diabetes, 2012).

	Type 1	Type 2
Age of onset	Usually during childhood or puberty	Commonly over age 35
Nutritional status at time of onset	Commonly undernourished	Obesity usually present
Prevalence	5% to 10% of diagnosed diabetics	90% to 95% of diagnosed diabetics
Genetic predisposition	Moderate	Very strong
Defect or deficiency	β cells are destroyed, eliminating the production of insulin	Inability of β cells to produce appropriate quantities of insulin; insulin resistance; other defects

Figure 2: Comparison Between Type1 and Type 2 Diabetes (Richard A. Harvey, 2013)

Type 1 diabetes: Most common in children and adolescent. It is characterized by suppression of insulin secretion due to autoimmune attack of β cells of the pancreas. β cell destruction occurs due to complex dialogue between invading or resident macrophages and T cells which release chemokines and cytokines in islet of Langerhans. This dialogue is developed according to host genetic background, age, environmental stimuli such as viral infection, diet, and others. A loss in function of β cell leads to failure of pancreas to respond to glucose and this condition requires an exogenous insulin to prevent sever hyperglycemia.

Type 2 diabetes: Accounts for more than 90% of cases. It is characterized by lack of sensitivity of target organs (liver, muscles, adipose tissue) to insulin. Hence, β cells retain

some function but insulin levels are insufficient to maintain normal glucose levels and this due to insulin resistance. Furthermore, type 2 diabetes is influenced by age, genetic factors, obesity, and insulin resistance rather than autoimmune response (Richard A. Harvey, 2013).

3. Diabetic Complications:

a. Microvascular Complications:

- Diabetic retinopathy: it is a microvascular complication that can affect retina, macula or both resulting in vision disability and blindness. Severity of diabetic retinopathy varies between non-proliferative, proliferative and highly proliferative depending on the abnormal growth of new vessels. Prevalence of developing retinopathy depends on severity and duration of hyperglycemia. Therefore, under poor glycemic control in diabetic patients it is frequently seen an impairment of retinal blood flow, high inflammatory cell adhesion to retinal blood vessels, and capillary blockage which can lead to hypoxia and destroy the retina (Kohner, 2003).
- Diabetic nephropathy: it is the main cause of renal failure in diabetic patients and it is characterized by albuminuria. Moreover, diabetic nephropathy is associated with pathological changes in the kidney which include thickening of glomerular basement membrane, increased glomerular filtration rate, microaneurysm formation, mesangial nodule formation and other changes. The risk factors that contribute to diabetic nephropathy are prolonged hyperglycemia, hypertension, and obesity (Cade, 2008).
- Diabetic neuropathy: can result in peripheral or autonomic neuropathy. Patients with autonomic neuropathy mainly suffer from cardiovascular autonomic dysfunction which is characterized by disruption in heart rate and vascular control (Vinik et al., 2003).

However, diabetes associated with peripheral neuropathy is featured by disorders affecting lower limb sensation which can be accompanied with impaired vascular function leading to foot ulceration (Cade, 2008).

b. Macrovascular Complications:

- Cardiovascular disease (CVD): is considered as the leading cause (70%) of death in type 2 diabetes. Diabetic patients have 4-fold higher risk of developing CVD than people with no diabetes. Moreover, people with diabetes have a 5-fold greater risk for a first myocardial infarction (MI) and a 2-fold higher risk for a recurrent MI compared to people who are not diabetic and previously had an MI. And it is well known that people with particularly type 2 diabetes are prone to developing CVD due to risk factors such as dyslipidemia, hypertension, obesity (Cade, 2008).

4. Macrophages and Diabetes:

There is growing evidence that inflammatory processes are involved in the onset and progression of diabetic complications. Among various immune cells, macrophages are implicated in in the development of diabetic complications such as retinopathy, neuropathy, nephropathy, and atherosclerosis (Tesch, 2007). For example, the development and progression of atherosclerosis in diabetes is associated with inflammatory cells such as monocyte that migrates into damaged endothelial cells and differentiate into macrophages ingesting oxidized LDL and forming foam cells. Foam cells attracts T lymphocytes and stimulate the proliferation of macrophages. The net result of these events is formation

atherosclerotic fatty streak (Cade, 2008). Moreover, macrophages are important in wound healing so impairment in the macrophage transition from proinflammatory M1 to anti-inflammatory M2 can result in deficiency of healing. Previous study showed that macrophages from diabetic mice exhibited dysregulation in M1/M2 phenotype which contributed to impaired healing of diabetic wound.(Mirza & Koh, 2011)

C. The Kallikrein-Kinin System:

1. Components of the Kallikrein-Kinin System:

The KKS is made up of large proteins (Kininogen), small polypeptides (Bradykinin and Kallidin), and group of enzymes that activate and deactivate the compounds formed (JN, 2012).

a. Proteins:

Kininogens are the substrates by which a kinin is released by the action of kallikrein enzyme. Kininogens are found in plasma, lymph, and interstitial fluid. In mammals kininogens are synthesized in the liver and occur predominantly in the plasma (Katzung, n.d.). There are two mammalian kininogens that act as precursor for kinins which are high molecular weight kininogen (HMWK (90-120kDa)) and low molecular weight kininogen (LMWK (50-68kDa)).

Kininogens are assembled by alternative splicing resulting in identical N-terminal but different C-terminal (Fig.3). Both LMWK and HMWK are encoded by a single gene present on chromosome 3. This gene consists of 11 exons and 10 introns. At the N-

terminal exons 1 to 9 codes for the heavy chain and contain 3 domains. Whereas exon 10 has the domain 4 which includes bradykinin sequence and 12 amino acids. Up to this point both kininogens share the same amino acid sequence. Upon alternative splicing of the gene transcript, two distinct C-terminal light chains are obtained: LMWK possess exon 11 that contains a single D5 domain (D5L), while HMWK has a D5 domain (D5H) along with a D6 domain. Domains (D1, D2, and D3) in LMWK and HMWK are cystatin like domains. D2 and D3 are cysteine protease inhibitors whereas D1 is inactive. D5H domain found in HMWK is rich with negatively charges histidine residues which bin to endothelial cells. And D6 domain in HK has pre-kallikrein and factor XI binding sites. However, domain D5L function remains unclear. Thus, the difference in C-terminal accounts for the difference in the molecular weight between HMWK and LMWK. (Lalmanach et al., 2010)

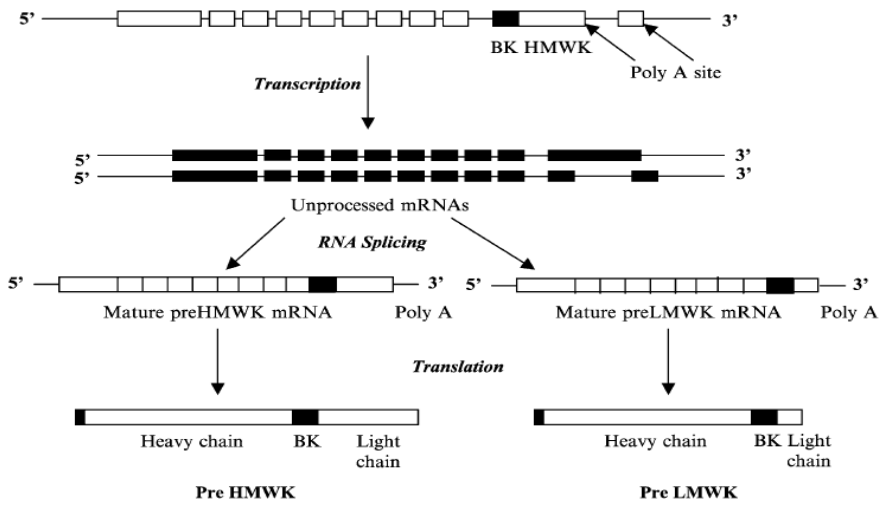

Figure 3: Transcription and Translation of Kininogen Gene (Joseph & Kaplan, 2005)

b. Enzymes:

Kallikrein are serine proteases possessing active sites and catalytic properties like those enzymes which are trypsin, plasmin, and elastase. In mammals there are two major kallikrein which are plasma kallikrein and tissue kallikrein.

Plasma Kallikrein is synthesized in the liver as inactive enzyme plasma pre-kallikrein. The zymogen plasma pre-kallikrein is a single polypeptide made up of 19 amino acids. While the active enzyme plasma kallikrein which circulates in blood is a single polypeptide chain of 619 amino acids (Bryant & Shariat-Madar, 2009).The activation of pre-kallikrein to kallikrein occurs by Hageman factor (factor XII), trypsin, or possibly kallikrein itself.

Carboxypeptidase also known as kininase I deactivates bradykinin and kallidin by removing arginine at their C-terminus. Kininase II its identical to angiotensin converting enzyme (ACE) it inactivates kinins by cleaving two amino acids phenyl-alanyl and arginine at the C-terminal.(Katzung, n.d.)

c. Kinins:

There are three kinins that have been identified in mammals which are: bradykinin (BK), kallidin (lysylBK), and methionyllysyl BK. Each kinin constitutes BK in its structure (Fig.4). It is important to note that plasma kallikrein cleaves HMWK to form bradykinin while tissue kallikrein cleaves LMWK to produce kallidin. And kallidin can be further transformed to BK by the action of plasma enzyme which is aminopeptidase.(JN, 2012)

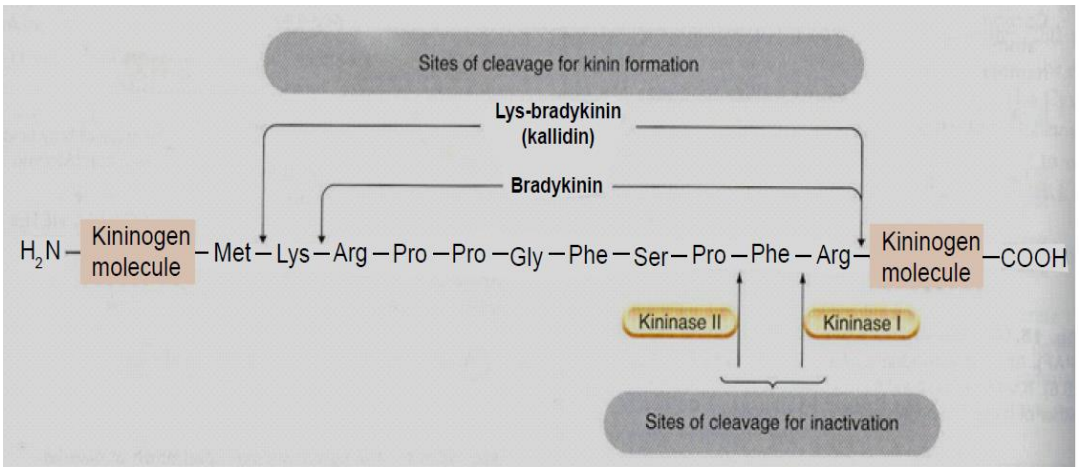

Figure 4: Amino Acid Sequence of Kinins (JN, 2012)

2. Bradykinin and its Formation:

Bradykinin is a nonapeptide that possess potent pro-inflammatory properties and it can elevate vascular permeability causing vasodilation.

The plasma pathway by which bradykinin is formed is closely related to the pathways of coagulation, fibrinolysis, and inflammation (Fig.5) (Joseph & Kaplan, 2005). As noted previously plasma prekallikrein is activated by Hageman factor (FXII clotting factor involved in intrinsic/contact pathway of coagulation). The contact system is comprised of two zymogens, FXII and prekallikrein, and a cofactor, high-molecular weight kininogen (HMWK) (Fredenburgh et al., 2017). FXII is usually inactive in plasma but when it contacts with negative surface such as collagen, basement membrane, bacterial lipopolysaccharides and urate crystals it changes conformation and become active FXIIa. The enzymatically active FXIIa has two substrates plasma pre-kallikrein and FXI. Thus, it will initiate two pathways:

- The cleavage of HMWK bound prekallikrein to kallikrein. The formed kallikrein can activate FXII to FXIIa which in turn produce more kallikrein. Moreover, kallikrein will cleave HMWK releasing bradykinin.
- The activation of FXI to FXIa resulting in fibrin formation (Ekdahl et al., 2016).

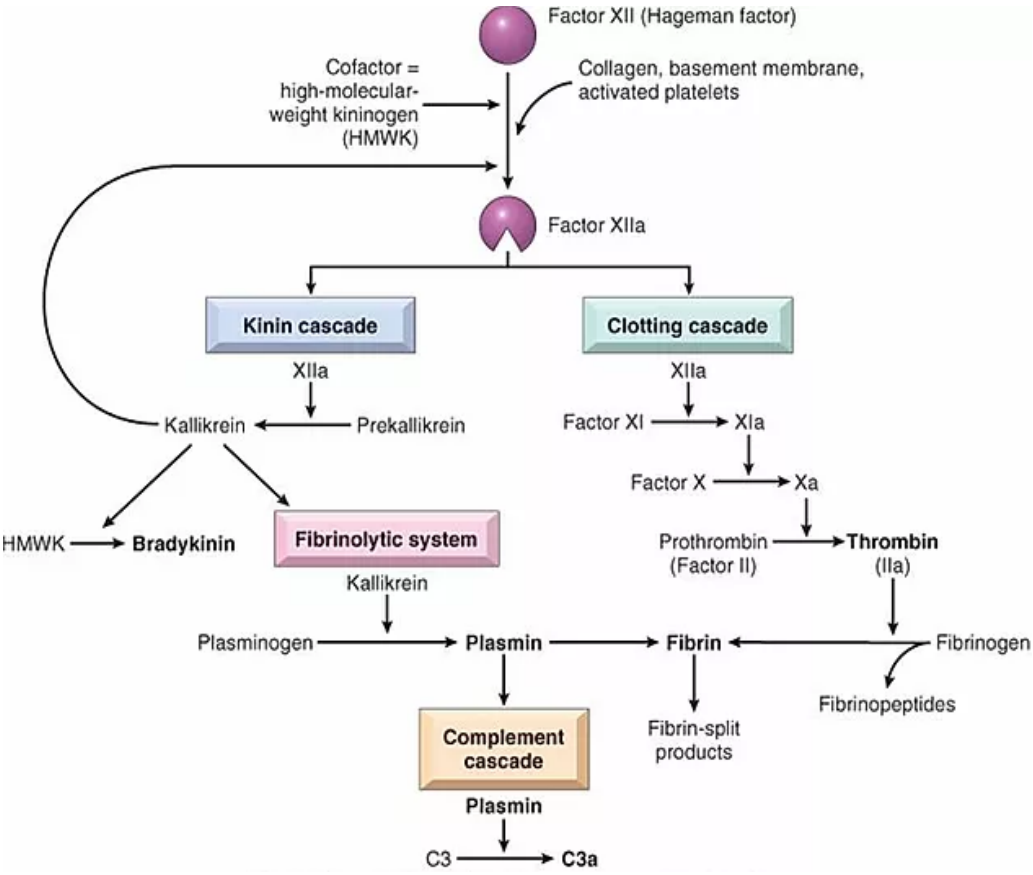

Figure 5: Plasma Pathways Activated by Hageman Factor And Formation of Bradykinin (Robbins Basic Pathology - 9th Edition, n.d.)

3. Receptors and their Signaling Pathways:

a. Bradykinin Receptors:

The action of bradykinin is mediated by two distinct subtypes of GPCRs which are B1R and B2R. B2R is constitutively expressed by wide range of cells such as endothelial cells, nociceptive fibers, and innate immune cells such as macrophages and dendritic cells. Although, B1R presence is limited in most tissues in normal conditions, its transcription is upregulated by proinflammatory cytokines such as (IL-1β, TNF-α, and IFN-γ) (Scharfstein, 2016). Example of cells that showed an upregulation of B1R after stimulation with proinflammatory cues are macrophages, neutrophils, mast cells, sensory C-fibers, epithelial cells, fibroblasts, smooth muscle, and cardiac cells (Leeb-Lundberg et al., 2005).

B1R is activated by metabolites generated by the proteolytic removal of the C-terminal arginine from intact BK such as des-Arg9-BK, Lys-des-Arg9-BK and des-Arg10-kallidin. In contrast, B2R responds to BK and Lys-BK (Marceau et al., 2020).

B1R participates in chronic inflammation and plays an important role in diseases with a strong immune component such as rheumatoid arthritis, multiple sclerosis, septic shock and diabetes. On the other hand,B2R is implicated in vasodilation, vasoconstriction, hypotension, pathogenesis of asthma through bronchoconstriction, acute inflammation, and acute visceral/somatic pain (Golias et al., 2007).

b. Bradykinin Signaling Pathways:

Kinins signaling through B1R or B2R activates several second messengers depending on the cell type. For example, in endothelial cells activation of B2R initiates the stimulation of PLCB which hydrolyzes PIP2 in to IP3 and DAG. IP3 will induce the release of Ca^{2+} stores from endoplasmic reticulum thus increasing cytosolic Ca^{2+}. Elevation of Ca^{2+} mediates the stimulation of eNOS and PLA2 that respectively release NO and PG. These mediators will activate cGMP and cAMP in VSMCs resulting in vasodilatory effect. Whereas, DAG will recruit PKC leading to activation of MAPKs, c-fos expression and phosphorylation. B1R has similar signal transduction pathway as B2R; however, Ca^{2+} levels vary between them (Fig.6) (JN, 2012).

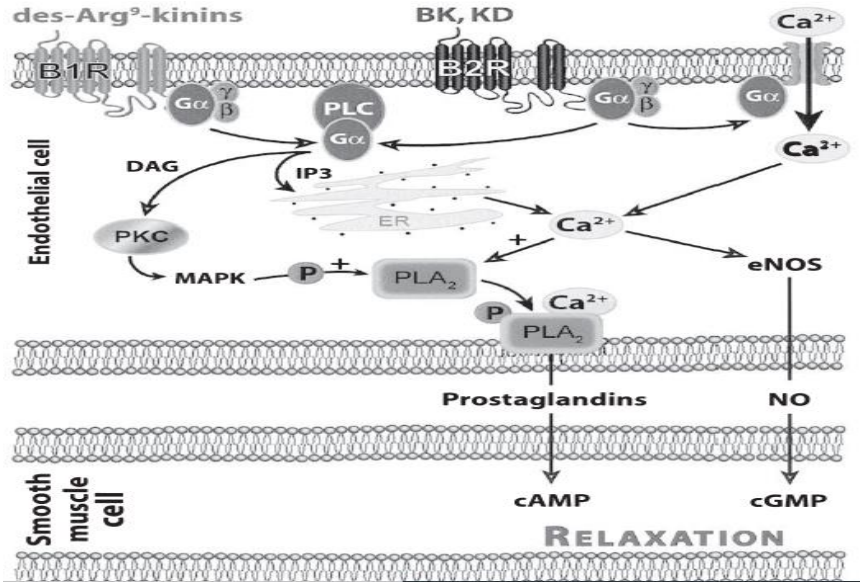

Figure 6: Downstream Signaling Pathways of B1R and B2R (JN, 2012)

c. Protease Activated Receptors (PARs):

PARs are class A GPCR with currently four members which are PAR1, PAR2, PAR3, and PAR4. PARs are extensively expressed in vascular, immune cells, epithelial cells, astrocytes as well as neurons. And they play critical role in homeostasis, inflammation, thrombosis and cancer progression (Soh et al., 2010a).

Although PARs are GPCRs they exhibit unusual activation mechanism. Proteases cleave the N-terminal of PAR at specific site so a new N- terminal is formed which acts as tethered ligand and binds to extracellular loop 2 of the receptor (Heuberger & Schuepbach, 2019).

PAR1, PAR3, and PAR4 are mainly activated by the coagulation enzyme thrombin. Thrombin can bind to PAR1/3 since they exhibit hirudin like site. PAR4 lacks this site; however, PAR4 activation by thrombin appears to be due to PAR3 that acts as a cofactor. On the other hand, PAR2 is a target for the serine protease enzyme trypsin.
In fact, the activation of PAR1 and PAR2 have been most extensively studied. These two can also be activated by kallikreins, factor Xa, plasmin, activated protein C, neutrophil elastase, and neutrophil protease 3 (Fig.7) (Arora et al., 2007a).

Receptor	*Tethered ligand*	*Activating proteases*	*Signalling effectors*	*Signal termination*
PAR_1	SFLLRN	Thrombin	G_q	Phosphorylation
		TF-VIIa-Xa or Xa	G_i	β-Arrestins
		APC-EPCR	$G_{12/13}$	Internalization
		Trypsin	Hsp90	Degradation
		Plasmin	Creatine kinase	
		MMP-1		
		Granzyme 1	Zyxin	
PAR_2	SLIGKV	Trypsin	G_q	Phosphorylation
		Tryptase	G_i	β-Arrestins
		TF-VIIa	$G_{12/13}$	
		TF-VIIa-Xa	β-Arrestins	
		Matriptase (MT-SP1)	Jab1	
		Bacterial gingipains		
		Kallikreins		
		Granzyme A		
PAR_3	TFRGAP	Thrombin	G_q	?
PAR_4	GYGQV	Thrombin	G_q	Internalization
		Trypsin	$G_{12/13}$	
		TF-VIIa-Xa		
		Plasmin		
		Cathepsin G		
		Bacterial ginginpains		
		Killikreins		
		MASP-1		

Figure 7: PARs and its Activating Proteases (Soh et al., 2010b)

d. Protease Activated Receptors Signaling:

PARs are known to initiate several major signaling pathways such as signaling via heterotrimeric guanylyl nucleotide binding proteins (G protein), or via beta arrestin pathway, or through transactivation of different receptors.

- Signaling via G-protein:

As any GPCR PARs signal via variety of G-proteins: Gq, Gi and G12/13 (Fig.8). The receptor acts as a ligand triggering guanin nucleotide exchange factor, this allow the replacement of GDP by GTP in Gα subunit. As a result, Gα dissociates from its tight binding to Gβγ dimer. Then each of Gα and Gβγ can independently interact with downstream signaling effectors.

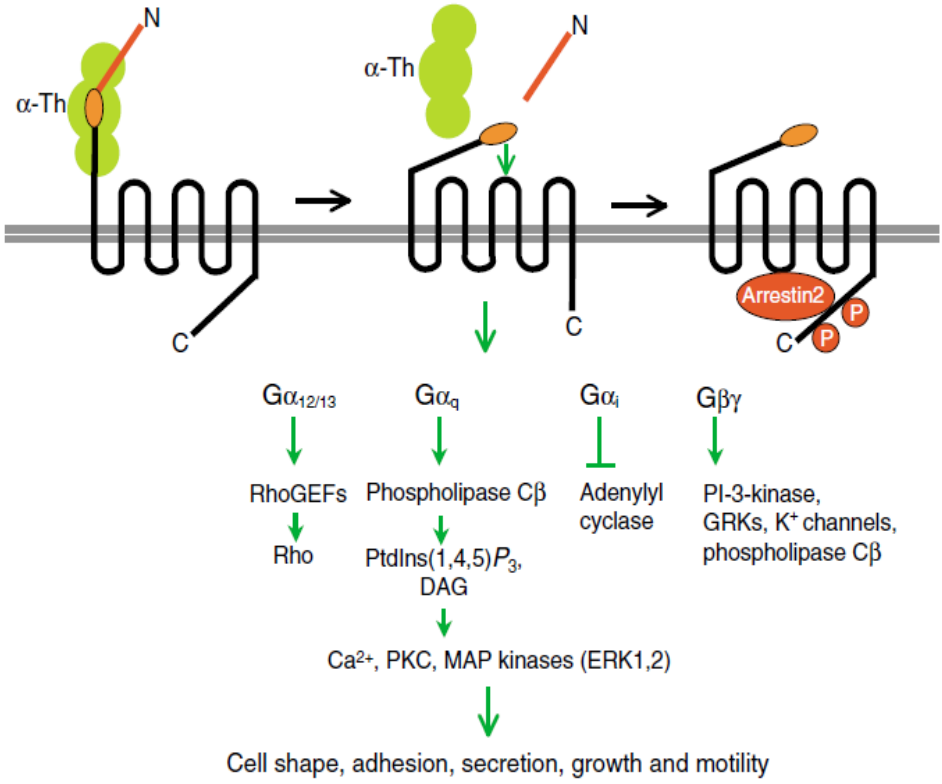

Figure 8: PARs Signaling via G-proteins (Arora et al., 2007b)

- Signaling via Beta arrestin:

In addition to signaling via G proteins PARs can use other strategy to modulate intracellular signaling. This strategy involves β-arrestin 1 and β- arrestin 2 which can lead to desensitization and endocytosis or can act as scaffold protein forming complex with a range of effector molecules such as MAPK, PI3K, or Src. The coupling of PARs to G protein or β-arrestin depends on the receptor conformational changes triggered by the ligand.

- Signaling via transactivation:

PARs transactivation of other receptors can be done through various mechanisms (Fig.9). One of these mechanisms is the extracellular release of agonist like prostaglandins or EGFR ligands that in autocrine or paracrine way stimulate non-PAR receptors. Another mechanism is the intracellular kinase pathway (Src kinase) which can activate EGFR in a ligand independent pathway. In addition, PARs can impact other signal mediators directly through dimer formation or indirectly via transactivation of ion channel or TLRs (Gieseler et al., 2013).

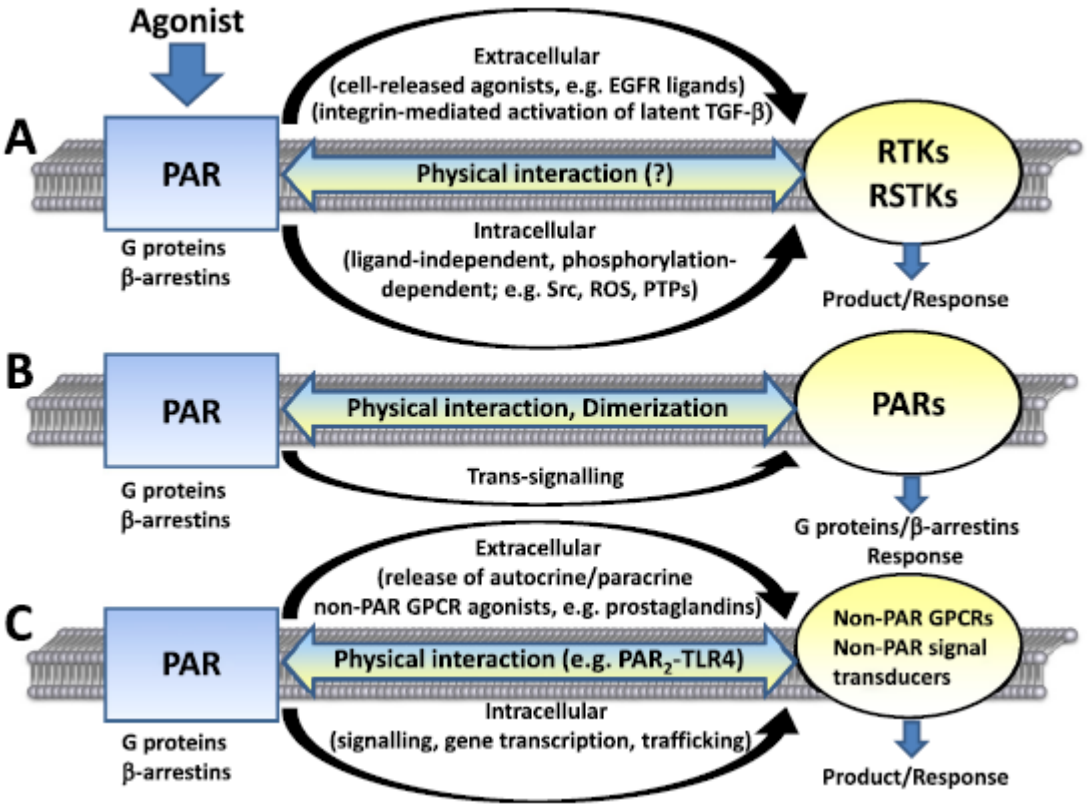

Figure 9: PARs Crosstalk with Other Receptors and Signal Transducers (Gieseler et al., 2013)

4. KKS Role in Diabetes:

The KKS has been recently involved in the development of diabetic microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular complications. Previous studies have shown that in type 1 diabetic patients high plasma PK level is correlated with elevated blood pressure and increased albumin excretion rate (A. A. Jaffa et al., 2003). Moreover, increased levels of circulating plasma prekallikrein was associated with carotid intima media thickness in type 1 diabetic subjects (M. A. Jaffa et al., 2016). Furthermore, continuous intravitreal injection of PK can lead to retinal vascular dysfunction and retinal thickening in diabetic rats (Clermont et al., 2011). In addition, it was shown that vitreous fluid from patients with diabetic retinopathy contains

increased amounts of plasma KKS components, including PK, coagulation Factor XII, and high molecular weight kininogen (Gao et al., 2008). Collectively, these evidences show that KKS components can act as a risk marker in diabetic complications.

CHAPTER II

AIM OF THE STUDY

Previous studies have shown that KKS is regarded as one of the main mechanisms implicated in the pathogenesis of inflammatory processes in diseases such as diabetes and its complications. Moreover, innate immune cells mainly macrophages are responsible for initiation and progression of inflammatory diseases (diabetes) and they contribute to microvascular and macrovascular complications. Therefore, we assumed that the KKS components can modulate macrophages' inflammatory responses under high glucose conditions.

In this study we aimed to:

1. Identify the impact of hyperglycemia on the expression of KKS components in BMDMs and Thp-1 macrophages.
2. Detect the effect of KKS on macrophages by:

i) Stimulating macrophages with BK and PK under normal and high glucose concentrations.

ii) Assessing cytokines release and the gene expression of KKS components, inflammatory and fibrotic markers.

3. Determine the role of proteases activated receptors in inflammation induced during diabetes by:

i) Treating macrophages with plasma kallikrein in presence and absence of PAR-1 and PAR-2 antagonists.

ii) Measuring gene expression of inflammatory and fibrotic markers.

CHAPTER III

MATERIALS AND METHODS

A. Bone Marrow Derived Macrophages (BMDMs):

1. Isolation and Culture:

Male C57BL/6J mice 8-10 weeks were obtained from Animal Facility at AUB and they were euthanized by exposure to CO_2. In brief, tibia and femur were removed and flushed with RPMI-1640 Medium (Sigma-Aldrich) supplemented with 10% FBS, 1% P/S, 1% L-glutamine and 1% HEPES. The flushed cells were centrifuged at 1500 rpm for 5 minutes. Then the supernatant was discarded, and the pellet was resuspended with Red Blood Lysis Buffer (RBL) (Sigma-Aldrich) diluted by PBS (Sigma-Aldrich) and incubated for 5 minutes at room temperature. After that, RPMI media was added above the RBL and it was centrifuged for 5 minutes at 1500 rpm. The obtained pellet was resuspended with the RPMI media then the cells were distributed into petri dishes in the presence of 20% L929 conditioned media (obtained from cell line that produces Macrophages Colony Stimulating Factor). Finally, the plates were incubated at 37˚C for 5 days allowing their differentiation into macrophages.

2. Treatment:

BMDMs were plated in six-well plates at density of 1,500,000 cells/well and incubated overnight for the macrophages to adhere to the plate. The next day, the supernatant was discarded and the cells were treated with two different glucose

concentrations: DMEM-low glucose (5.5mM glucose, Sigma-Aldrich) used as normal glucose medium; on the other hand, for hyperglycemic conditions DMEM-low glucose (5.5mM glucose, Sigma- Aldrich) was supplemented with sterile D- glucose to yield a concentration of 20mM. Furthermore, the cells were stimulated for 24 hours with 100 ng/ml LPS as positive control, 10^{-7}M BK and 2.5 ng/ml PK under high and normal glucose conditions. After 24 hours the supernatant was collected and preserved at -20 °C for ELISA whereas the plates were stored at -80°C for RNA extraction.

3. Gene Expression Measurement in BMDM:

a. RNA Extraction:

Total RNA was extracted using QIAzol (QIAGEN). Shortly, 500µl of QIAzol was added to each well and the cell lysate was collected in Eppendorf tubes. Then 100µl of chloroform was added to separate the aqueous phase from DNA and protein phases. Total RNA was dissolved in aqueous phase so 200µl of isopropanol was added for the RNA to precipitate. The quality and quantity of RNA was assessed using Nanodrop ND- 1000 Spectrophotometer (DeNovix DS-11 FX+). The ratios 260/280 and 260/230 were used to measure the purity of RNA and a ratio of 1.8-2 was accepted as pure RNA.

b. Reverse Transcription:

5 µg of RNA from each sample was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The reaction was held in T100^{TM} Thermal Cycle (BIO-RAD) machine in the following environmental conditions: 10 minutes at 25 °C, 2 hours at 37 °C, 5 minutes at 85 °C till it ends at 4 °C.

c. Quantitative Real Time Polymerase Chain Reaction:

The genes expression was quantified using CAPITAL^{TM} qPCR Probe Mix, 4x and carried on CFX384 Touch real time PCR Detection System (BIO-RAD). Following this protocol: polymerase activation at 95°C for 5 minutes, denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, Extension at 72°C for 30 seconds, and melt curve for 5 seconds. The gene expression level was calculated using $2^{-\Delta\Delta CT}$ method and normalized to the housekeeping gene GAPDH. The primer sequences used in the study are shown in table 1.

Table 1: Primers Sequence of Murine Genes

Murine Genes	Forward	Reverse
GAPDH	CGTCCCGTAGACAAAATGGTGAA	GCCGTGAGTGGAGTCATACTGGAACA
Bdkrb1	CCATCAGTCAGGACCGCTAC	AGGGACGACTTTGACGGAAC
Bdkrb2	CTGGGTGTTTGGAGAGGTGT	ACGAGCATCAGGAAGCAGAT
F2RL1	TGCTTTGCTCCTAGCAACCT	CAGAGGGCGACAAGGTAGAG
F2R	TGCATCGATCCGTTGATTTA	TGCAACTGTTGGGATCAGAG
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
TNF-α	CTAGTTTCCTGGTCTGGAGAAGATC	CCCTCTCCACCAGTCTCCTCTA
CTGF	AGATGGTGCACCCTGTGTCT	CCAGGCAAGTGCATTGGTAT
L-gal3	ACAAGTCCTGGTTGAAGCTG	GCTGGTGAGGGTTATGTCAC

KNG 1	GCCAGGGAGCAAGAAGAGAG	CCCATGCTTATGACCACGGT
TBXA2R	CCTCCTGCTCAACACCGTTA	AACCATCATCTCCACCTCGC
IL-1β	GCTGCTTCCAAACCTTTGAC	GCTGCTTCCAAACCTTTGAC
Cox-2	TTGGAGGCGAAGTGGGTTTT	GGTAGGCTGTGGATCTTGCA

4. Enzyme Linked Immunoassay (ELISA):

IL-6 levels in BMDM supernatants were measured using Mouse IL-6 ELISA Kit (Invitrogen) according to manufacturer's instructions. Briefly, 100µl of captured antibody was distributed in 96-well plate and kept overnight at 4°C. After washes, 200µl of 5X ELISA Diluent was added to each well and incubated at room temperature for 1 hour. When incubation was done the plate was washed and 100µl/well of each the samples and standard were pipetted. Then the plate was incubated for 2 hours. Followed that the plate was washed thoroughly and 100µl of diluted Detection Antibody was added per well. After that, the plate was incubated for 1 hour at room temperature. 1 hour later washes were done and 100µl/well of diluted Streptavidin-HRP was distributed. The plate was kept at room temperature for half an hour. Finally, 100µl of TMB solution was added and after 15 minutes the assay was stopped using 50µl/well of Stop Solution. Plate was read at 450nm using spectrophotometer.

B. THP-1 Macrophages:

1. Cell Culture:

THP-1 cells were cultured in 75 cm^2 flask in RPMI-1640 Medium (Sigma-Aldrich) supplemented with 10% FBS, 1% P/S, and 1% HEPES ensuring that its density do not exceed 1,000,000 cells/ml. Moreover, the cells were incubated at 37°C throughout the experiments.

2. Treatment:

a. High and Low Glucose Concentrations:

THP-1 cells were stimulated with 25 nM PMA then distributed in 6-well plates at a concentration of 1,500,000 cells/well. The plates were incubated at 37°C for 72 hours to allow differentiation of monocytes in to macrophages. After 72 hours the cells were starved using DMEM-low glucose (5.5 mM glucose, Sigma-Aldrich) containing 1% FBS, 1% P/S. Then, the plates were incubated for 24 hours. The next day, the media was changed by DMEM-low glucose (5.5mM glucose and 1% P/S with no FBS) representing low glucose conditions. However, for high glucose conditions the DMEM-low glucose media was supplemented with D-glucose to achieve a concentration of 20 mM.

b. Stimulation with LPS, BK, and PK:

After treating with low and high glucose media, the cells were stimulated for 24 hours with 100ng/ml LPS as positive control,10^{-7}M BK, and 2.5ng/ml PK. Finally, media was collected for ELISA and stored at -20°C while the plates were preserved at -80°C for RNA extraction.

c. PAR-1 and PAR-2 Antagonists:

PAR-1 antagonist (SCH530348) and PAR-2 antagonist (GB83) were purchased from Axon Medchem BV, Cedarlane and used at concentrations of 0.75 and 2μM, respectively. Macrophages were pretreated with PAR-1 and PAR-2 antagonists for 30 minutes then stimulated with 2.5ng/ml PK for 24 hours under low and high glucose conditions. Next day, the plates were preserved at -80°C for RNA extraction.

3. Gene Expression Measurement in THP-1 Macrophages:

a. RNA Extraction:

Total RNA was extracted using QIAzol (QIAGEN). In brief, 500µl of QIAzol was added to each well and the cell lysate was collected in Eppendorf tubes. Then 100µl of chloroform was added to separate the aqueous phase from DNA and protein phases. Total RNA was dissolved in aqueous phase so 200µl of isopropanol was added for the RNA to precipitate. The quality and quantity of RNA was assessed using Nanodrop ND- 1000 Spectrophotometer (DeNovix DS-11 FX+). The ratios 260/280 and 260/230 were used to measure the purity of RNA and a ratio of 1.8-2 was accepted as pure RNA.

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5µg of RNA from each sample was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientifics). The reaction was held in T100^{TM} Thermal Cycle (BIO-RAD) machine in the following environmental conditions: 10 minutes at 25 °C, 2 hours at 37 °C, 5 minutes at 85 °C till it ends at 4 °C.

c. Quantitative Real Time Polymerase Chain Reaction:

The genes expression was quantified using CAPITAL^{TM} qPCR Probe Mix, 4x and carried on CFX384 Touch real time PCR Detection System (BIO-RAD) following this protocol: polymerase activation at 95°C for 5 minutes, denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, Extension at 72°C for 30 seconds, and melt curve for 5 seconds. The gene expression level was calculated using $2^{-\Delta\Delta CT}$ method and normalized to the housekeeping gene GAPDH. The primer sequences used in the study are shown in table 2.

Table 2: Primer Sequence of Human Genes

Human Genes	Forward	Reverse
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
Bdkrb1	TGGGTTTCCTCCTACCACTG	CTTGTCCTGCTGACCTCCTC
Bdkrb2	CTTCATGGCCTACAGCAACA	GCACACTCCCTGGTACACCT
F2RL1	TTGGTAAGGTTGATGGCACA	CCAGTGAGGACAGATGCAG
F2R	TCCGGATATTTGACCAGCTC	CAGGATGAACACAACGATGG
IL-6	CTTCGGTCCAGTTGCCTTCT	GAGATGCCGTCGAGGATGTA
TNF-α	CAGAGGGCCTGTACCTCATC	GGAAGACCCCTCCCAGATAG
CTGF	GGCCCAGACCCAACTATGAT	TCTCTTCCAGGTCAGCTTCG
L-gal3	AATTCCACACTGTGCCCTTC	TGTAAATCCGGGGCTTGTAG
KNG 1	GTCCTGCGAGTACAAGGGTC	CAGAGGTGGTTGGGGCTATG
TBXA2R	CTTCCTGCTGAACACGGTCA	GAGCCATCATCTCCACCTCG

4.Enzyme Linked Immunoassay (ELISA):

IL-6 levels in THP-1 supernatants were measured using Human IL-6 ELISA Kit (Invitrogen) according to manufacturer's instructions. Briefly, 100µl of captured antibody was distributed in 96-well plate and kept overnight at 4°C. After several washes, 200µl of 5X ELISA Diluent was added to each well and incubated at room temperature for 1 hour. When incubation was done the plate was washed for couple of times and 100µl/well of each the samples and standard were pipetted. Then the plate was incubated for 2 hours. Next, the plate was washed thoroughly and 100µl of diluted Detection Antibody was added per well. After that, the plate was incubated for 1 hour at room temperature. 1 hour later washes were done and 100µl/well of diluted Streptavidin-HRP was distributed. The plate was kept at room temperature for half an hour. Finally, 100µl of TMB solution was added and after 15 minutes the assay was stopped using 50µl/well of Stop Solution. Plate was read at 450nm using spectrophotometer.

C. Statistical Analysis:

All data were analyzed using GraphPad Prism8 Software and represented as mean ± SD. And Mann Whitney U test was carried out to detect any statistical significance between two experimental data sets. P values less than 0.05 were considered significant.

CHAPTER IV

RESULTS

A. Impact of Hyperglycemia and KKS Components on BMDMs:

1. The Effect of High Glucose on Gene Expression:

BMDM were exposed to high glucose (20mM) and normal glucose (5.5mM) concentrations for 24 hours. Then the gene expression of inflammatory, fibrotic and KKS components were assessed using RT-qPCR.

a. Effect of High Glucose on Inflammatory and Fibrotic Gene Expression:

High glucose treated BMDMs slightly increased the mRNA levels of CTGF, Gal-3 and IL-6 compared to normal glucose concentration. However, high glucose levels did not affect the gene expression of Cox-2 and IL-1β (Fig.10).

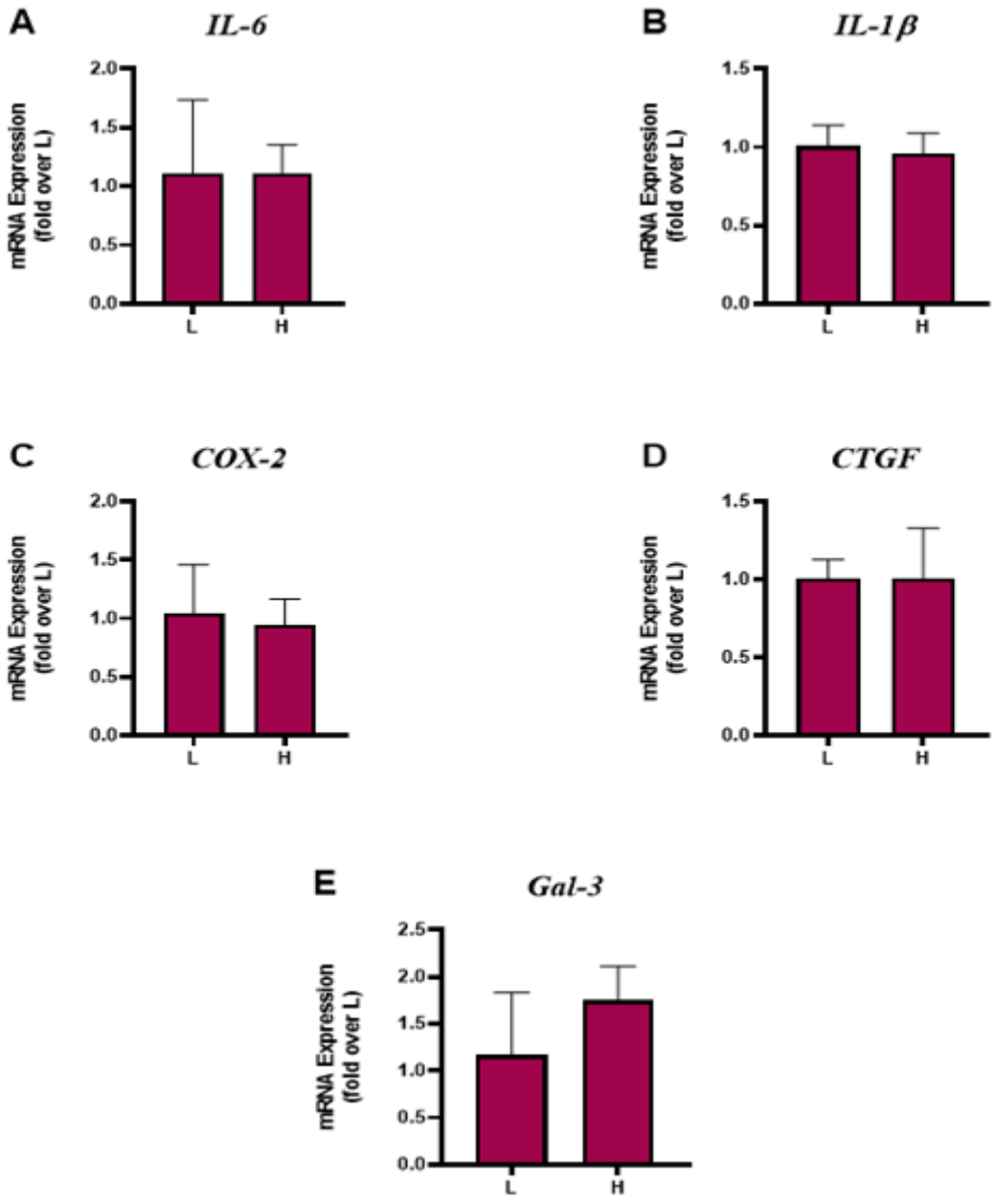

Figure 10. Measurement of Inflammatory and Fibrotic mRNA Levels Under High Glucose Conditions. BMDMs were treated with L (low glucose media 5.5mM) representing normal glucose condition. Also, BMDMs were exposed to H (high glucose media 20 mM) representing hyperglycemic condition. **A-E** RT-qPCR showing gene expression of **(A)**IL-6, **(B)** IL-1β, **(C)** Cox-2, **(D)** CTGF, **(E)** Gal-3. Data are represented as mean ± SD (N= 3). Mann- Whitney U test was performed.

b. Effect of High Glucose on mRNA Levels of KKS Components:

An insignificant increase in mRNA levels of B1R, PAR-2, KNG and TBXA2R was observed under hyperglycemic conditions compared to normal glucose concentration. On the other hand, the gene expression of B2R and PAR-1 was not influenced by high glucose concentration (Fig.11).

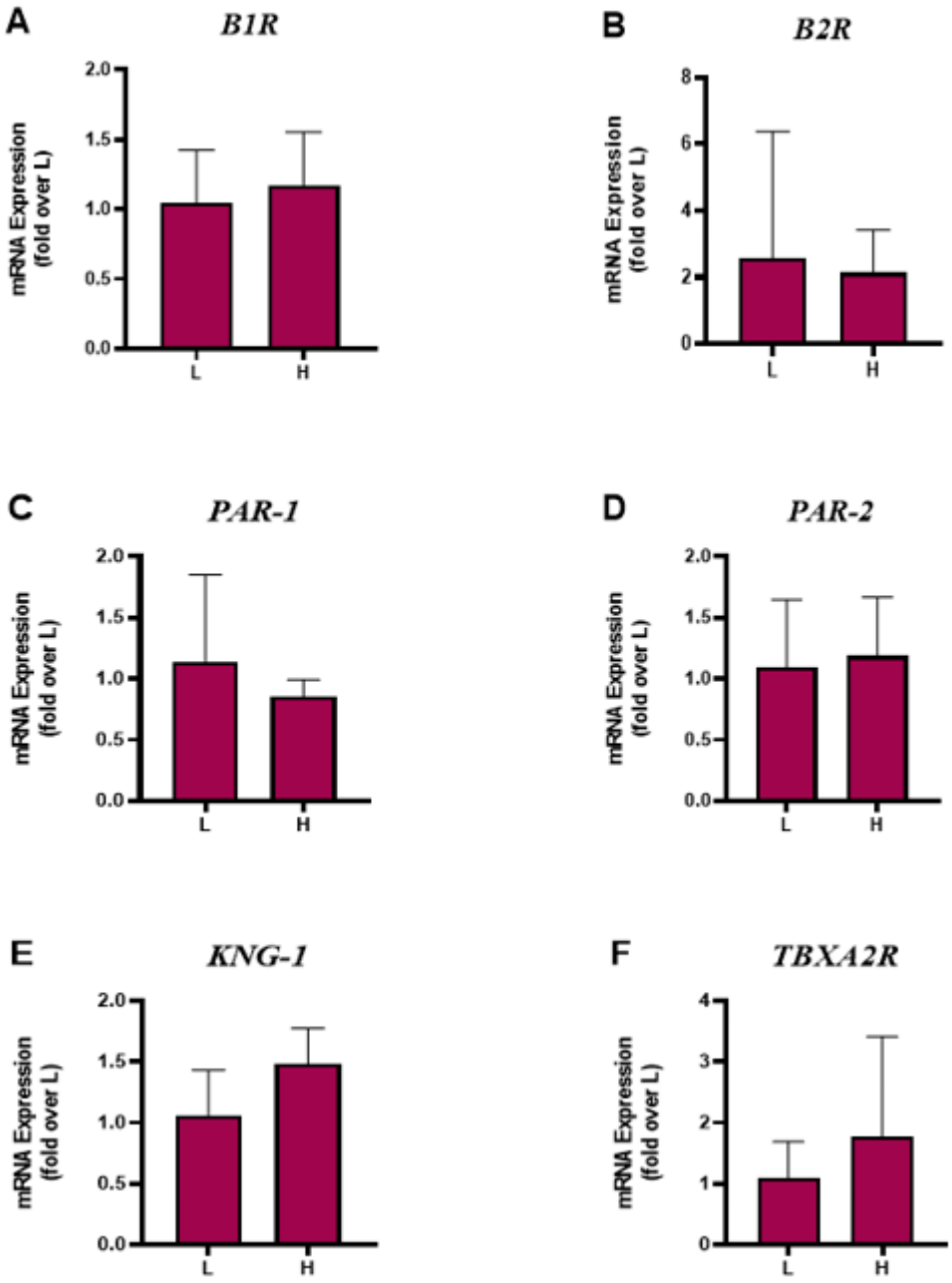

Figure 11. Exposure to High Glucose Levels Slightly Induced KKS Components Gene Expression. mRNA levels of (A) B1R, (B) B2R, (C) PAR-1, (D) PAR-2, (E) KNG, (F) TBXA2R. Data are represented as mean ± SD (N=3). Mann-Whitney U test was carried out.

2. The Impact of KKS Components on Gene Expression Under Normal and High Glucose Levels:

We next assessed gene expression of inflammatory, fibrotic and KKS components after stimulating BMDMs with LPS (100ng/ml), BK (10^{-7}M), and PK (2.5 ng/ml) under normal and high glucose concentrations.

a. Inflammatory Effect of BK and PK on BMDMs under Normal Glucose Conditions:

PK treated BMDMs significantly induced galectin-3 gene expression compared to unstimulated normal glucose treated BMDMs. Also BK was able to increase Gal-3 mRNA levels but slightly lower than PK. And both PK and BK insignificantly elevated CTGF gene expression. However, BK alone increased IL-1β by approximately 2 folds and minimally raised up Cox-2 and IL-6 mRNA levels under normal glucose conditions (Fig.12).

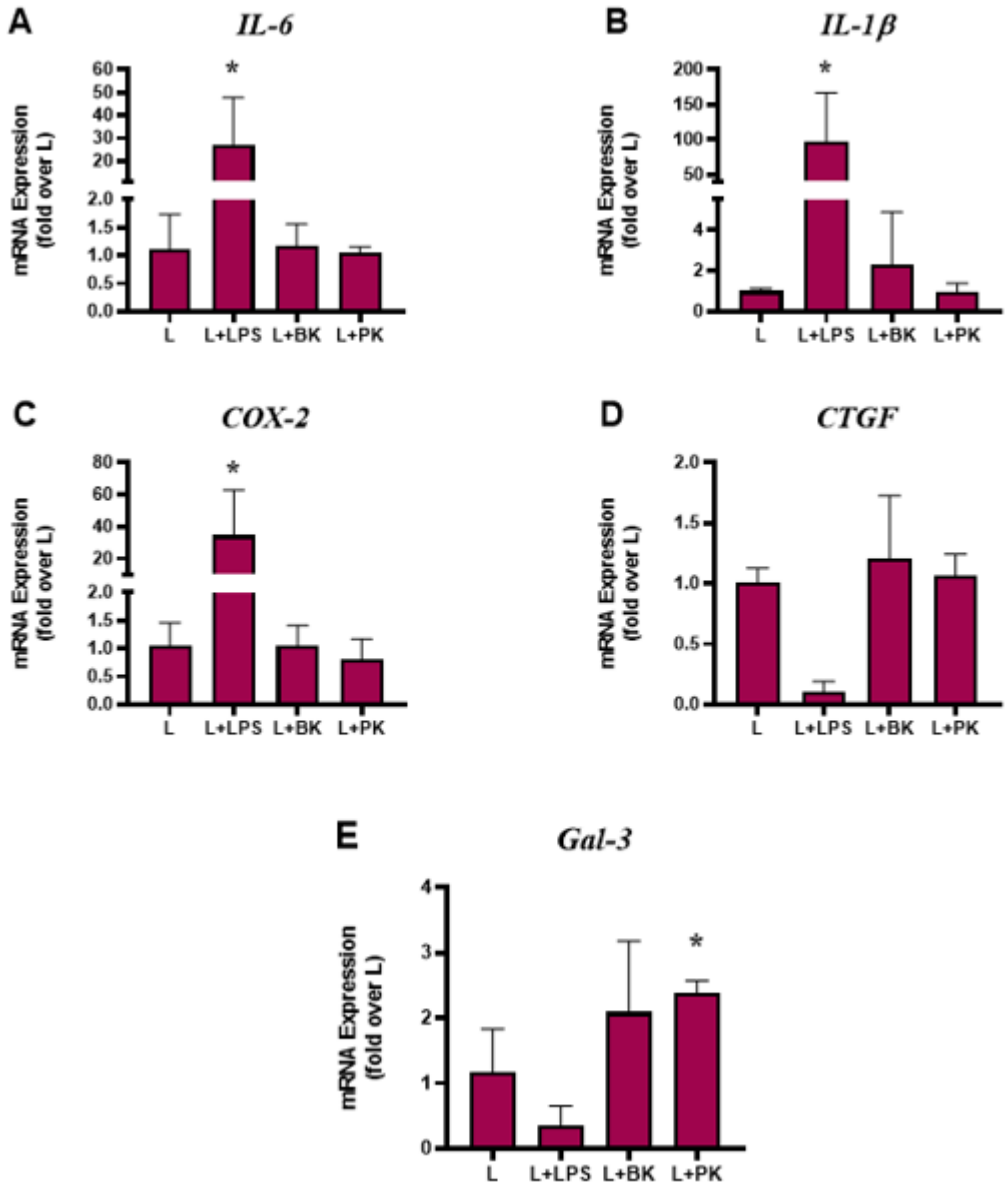

Figure 12. Effect of BK and PK on Inflammatory and Fibrotic Gene Expression Under Normal Glucose Conditions. A-E RT-qPCR showing gene expression of **(A)**IL-6, **(B)** IL-1β, **(C)** Cox-2, **(D)** CTGF, **(E)** Gal-3. Data are represented as mean ± SD (N= 3), * ($P<0.05$) compared to unstimulated control (L). Mann- Whitney U test was performed.

b. mRNA levels of KKS Components in Response to BK and PK Stimulation Under Normal Glucose Conditions:

B1R and PAR-2 gene expression was enhanced after stimulation with BK and PK compared to untreated BMDMs. However PAR-2 gene expression was more up-regulated in response to PK rather than BK stimulation. On the other hand, BK- treated BMDMs boosted B2R, PAR-1, KNG and TBXA2R mRNA levels (Fig.13).

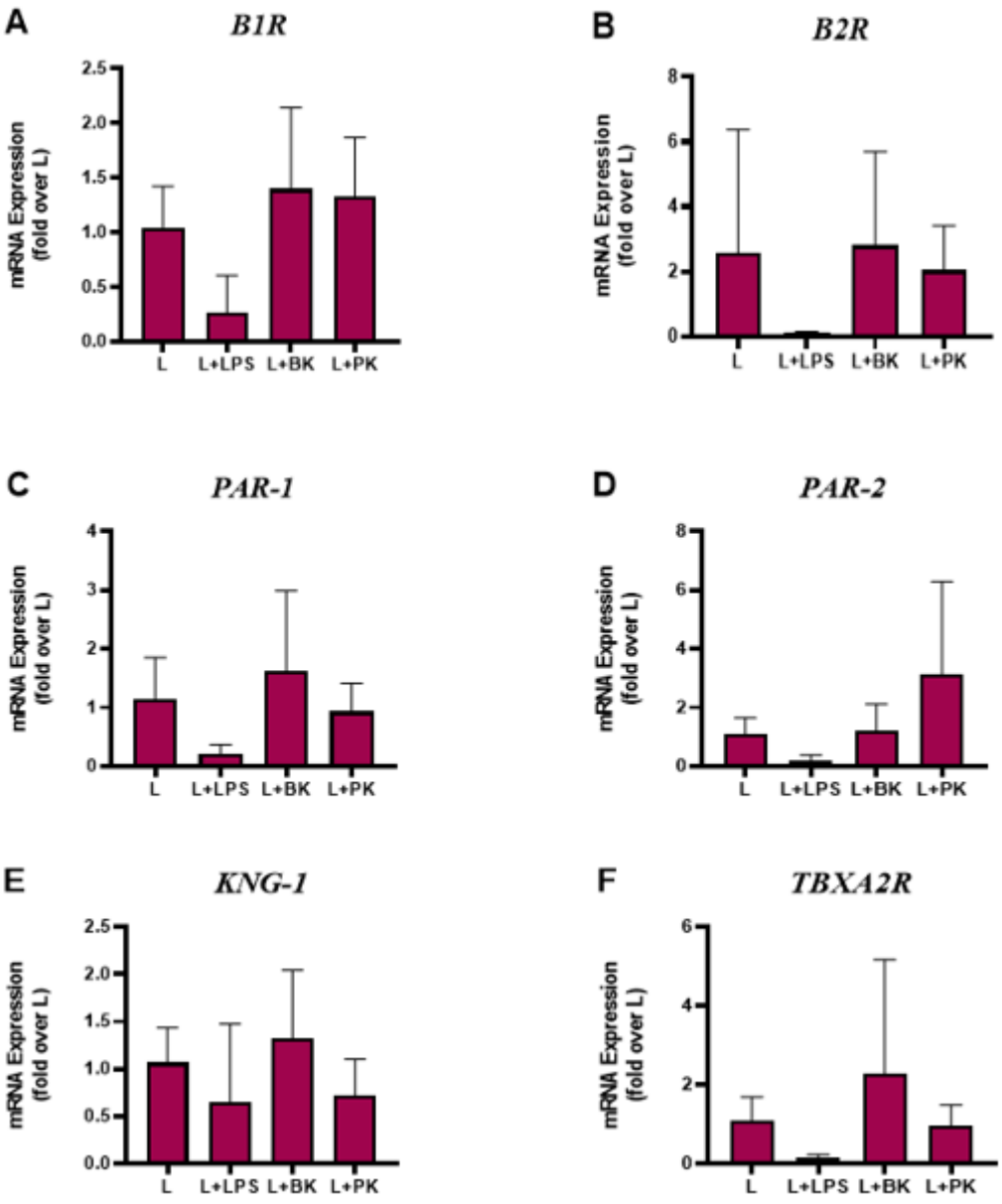

Figure 13. Elevation of KKS Components Gene Expression Upon Stimulation with BK and PK Under Normal Glucose Levels. mRNA levels of **(A)** B1R, **(B)** B2R, **(C)** PAR-1, **(D)** PAR-2, **(E)** KNG, **(F)** TBXA2R. Data are represented as mean ± SD (N=3). Mann-Whitney U test was carried out.

c. BK and PK Inflammatory Effect Under Hyperglycemic Environment:

Under high glucose concentration PK treated BMDMs significantly increased IL-6. In addition, it up-regulated Gal-3, and IL-1β mRNA levels. It also showed a negligible stimulation of Cox-2 and CTGF gene expression compared to unstimulated high glucose treated BMDMs. Moreover, BK barely elevated the gene expression of IL-1β (Fig.14).

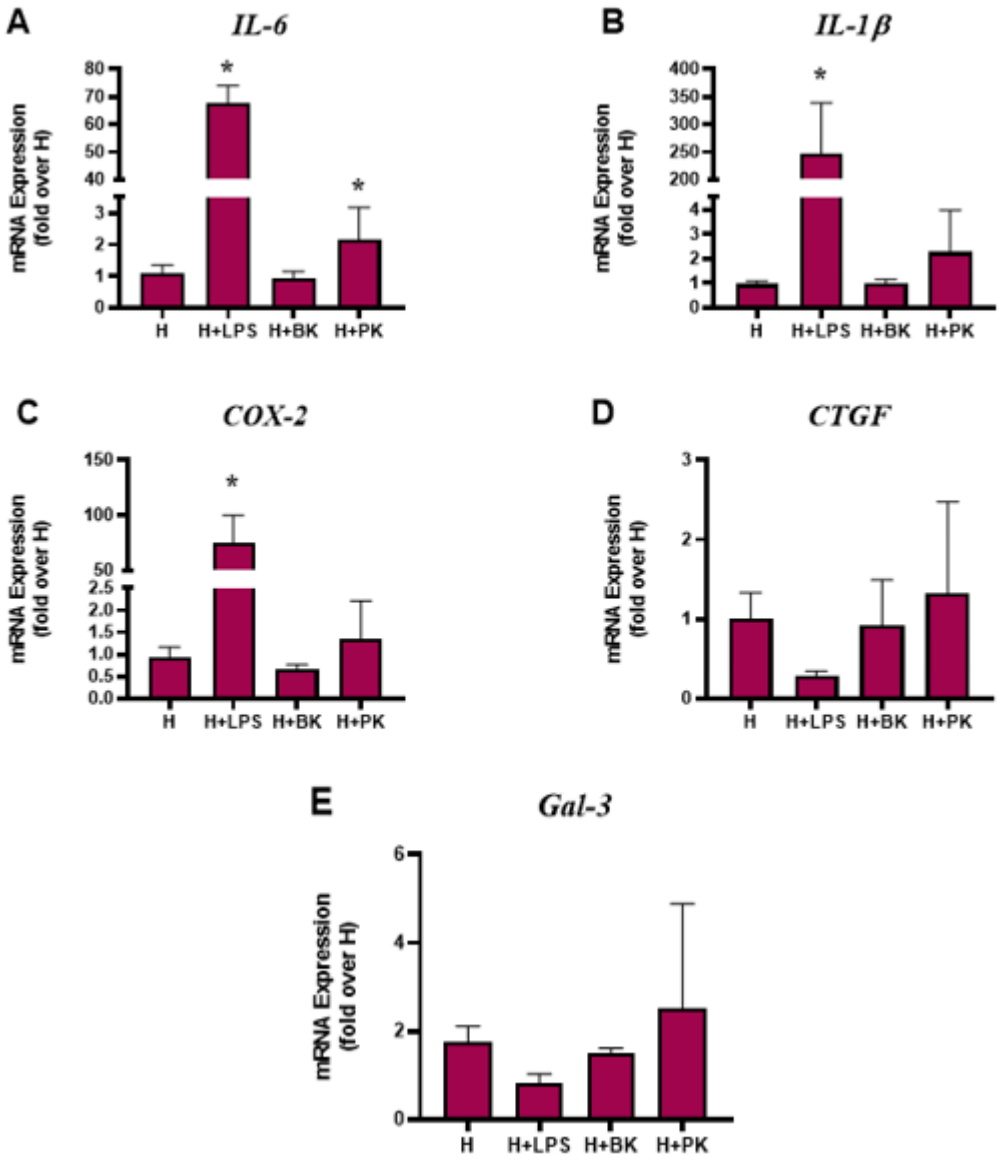

Figure 14. Mainly PK Induced BMDMs Inflammatory Response Under Hyperglycemic Conditions. A-E RT-qPCR showing gene expression of **(A)** IL-6, **(B)** IL-1β, **(C)** COX-2, **(D)** CTGF, **(E)** Gal-3. Data are represented as mean ± SD (N= 3), * ($P<0.05$) compared to unstimulated control (H). Mann- Whitney U test was performed.

d. mRNA Levels of KKS Components in High glucose Treated BMDMs and Stimulated with BK and PK:

B2R gene expression was elevated in response to PK and BK stimulation compared to high glucose treated BMDMs without stimulation. However, PK showed a greater increase in B2R expression than BK. Also B1R gene expression was induced in response to PK-treated BMDMs. Moreover, BK alone barely elevated PAR-1 mRNA levels compared to unstimulated cells. No increase was observed on mRNA levels of PAR-2, KNG, and TBXA2R in response to BK and PK (Fig.15).

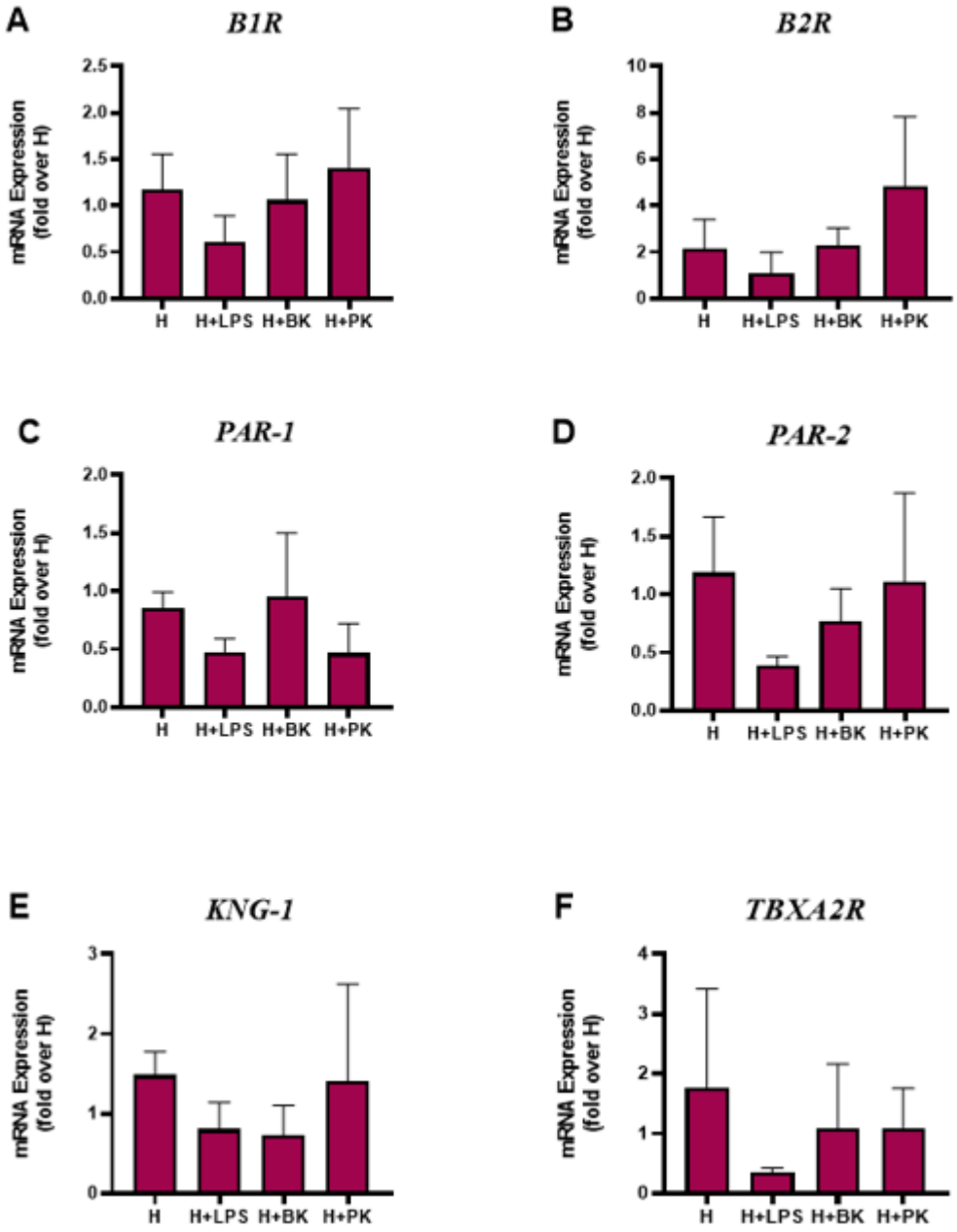

Figure 15. Stimulation with BK and PK Under Hyperglycemic Conditions Increased KKS Components Gene Expression. mRNA levels of **(A)** B1R, **(B)** B2R, **(C)** PAR-1, **(D)** PAR-2, **(E)** KNG, **(F)** TBXA2R. Data are represented as mean ± SD (N=3). Mann-Whitney U test was carried out.

B. Assessment of Pro-inflammatory Cytokine Release in BMDMs:

1. IL-6 Secretion in Response to Hyperglycemic Conditions:

As mentioned previously BMDMs were treated with normal glucose (5.5mM) and high glucose (20 mM) media for 24 hours. Then ELISA was performed to investigate the effect of high glucose on IL-6 production. And it was observed that high glucose did not stimulate IL-6 secretion (Fig. 16).

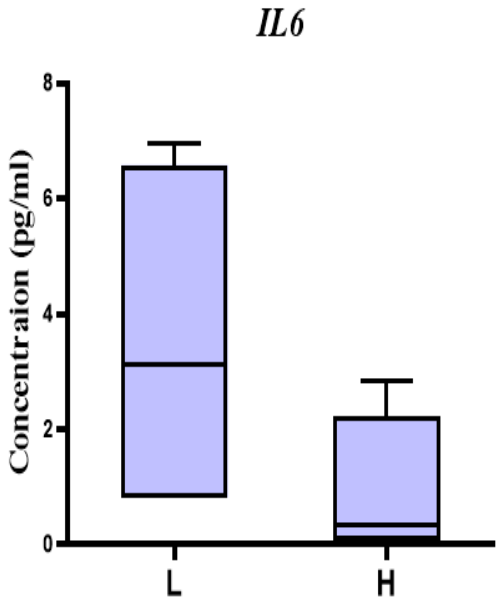

Figure 16. Hyperglycemia did not Promote IL-6 Secretion in BMDMs. IL-6 concentration expressed in pg/ml was measured in L (low glucose media representing normal conditions) and H (High glucose Conditions). Data are represented as mean ± SD (N=4). Mann-Whitney U test was carried out.

2. IL-6 Release in Response to KKS under Normal and High Glucose Conditions:

a. <u>BK and PK Stimulation under Normal Glucose Concentration:</u>

Examination of IL-6 production showed a significant increase in PK-treated BMDMs compared to unstimulated macrophages. Moreover, BK induced IL-6 release but its effect was lower than PK (Fig.17).

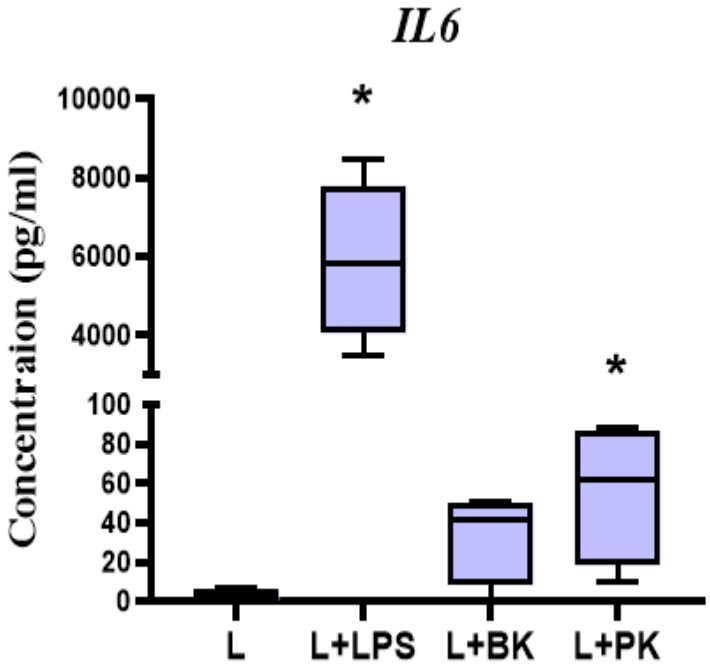

Figure 17: IL-6 Concentration in BK and PK- treated BMDMs under Normal Glucose Levels. Data are represented as mean ± SD (N=4), * ($P<0.05$) compared to unstimulated control (L). Mann-Whitney U test was carried out.

b. BK and PK Stimulation under High Glucose Concentration:

PK elevated the release of IL-6 compared to high glucose treated cells that were not stimulated. Furthermore, IL-6 production was induced in response to BK but to a lesser extent than PK stimulated BMDMs (Fig. 18).

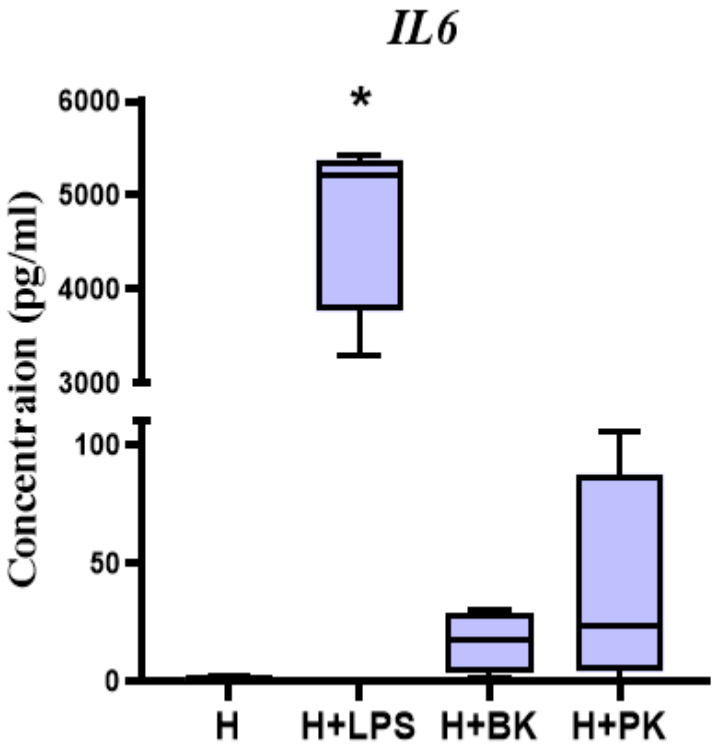

Figure 18: IL-6 Production in High Glucose Treated BMDMs in Presence of BK and PK Stimulation. Data are represented as mean ± SD (N=4), * ($P<0.05$) compared to unstimulated control (H). Mann-Whitney U test was carried out.

C. The Impact of Hyperglycemia and KKS on THP-1 Macrophages:

To assess whether PK and BK inflammatory effect is also relevant in human macrophages, we used THP-1 macrophages and quantified the gene expression of inflammatory, fibrotic, and KKS components by RT-qPCR under normal (5.5mM) and high glucose (20mM) conditions.

1. The Effect of High Glucose Treated THP-1 Cells on Gene Expression:

a. Inflammatory and Fibrotic Gene Expression under High Glucose Levels:

A negligible increase in mRNA levels of gal-3 was detected under high glucose concentration. On the other hand, the gene expression of il-6, TNF-α, and CTGF was not affected by exposure to high glucose (Fig.19).

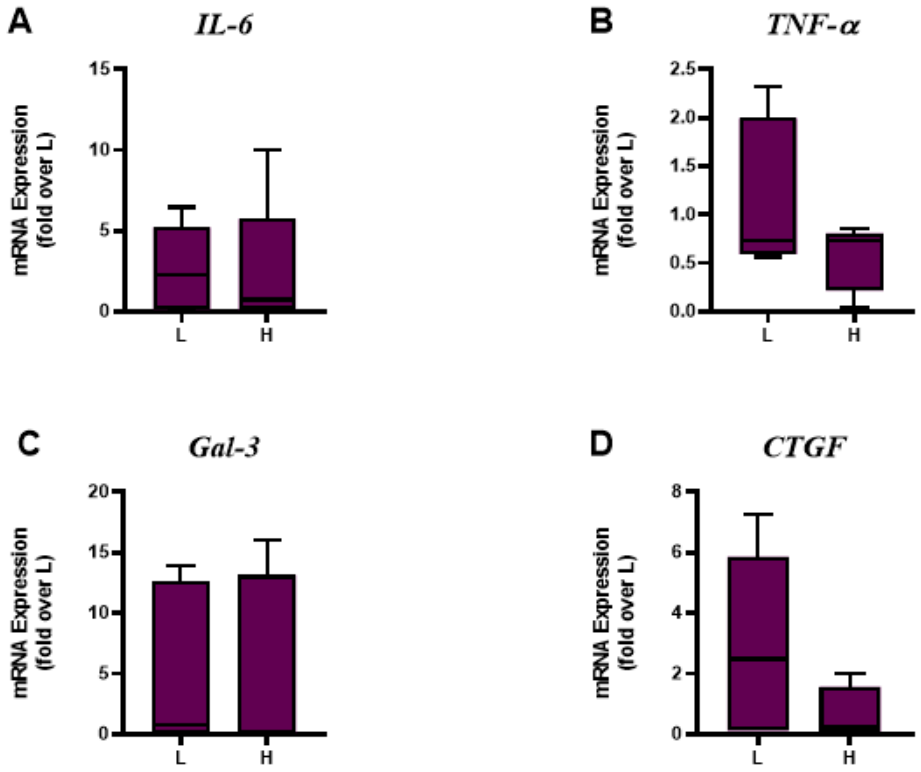

Figure 19. Hyperglycemic Effect on THP-1 Inflammatory Response. A-D RT-qPCR showing gene expression of **(A)** IL-6, **(B)** TNF-α, **(C)** Gal-3, **(D)** CTGF. Data are represented as mean ± SD (N= 5). Mann- Whitney U test was performed.

b. Gene Expression of KKS Components under High Glucose Levels:

High glucose concentration insignificantly enhanced mRNA levels of KNG and TBXA2R. However, the gene expression of B1R, B2R, PAR-1, and PAR-2 was not affected by hyperglycemic conditions (Fig.20).

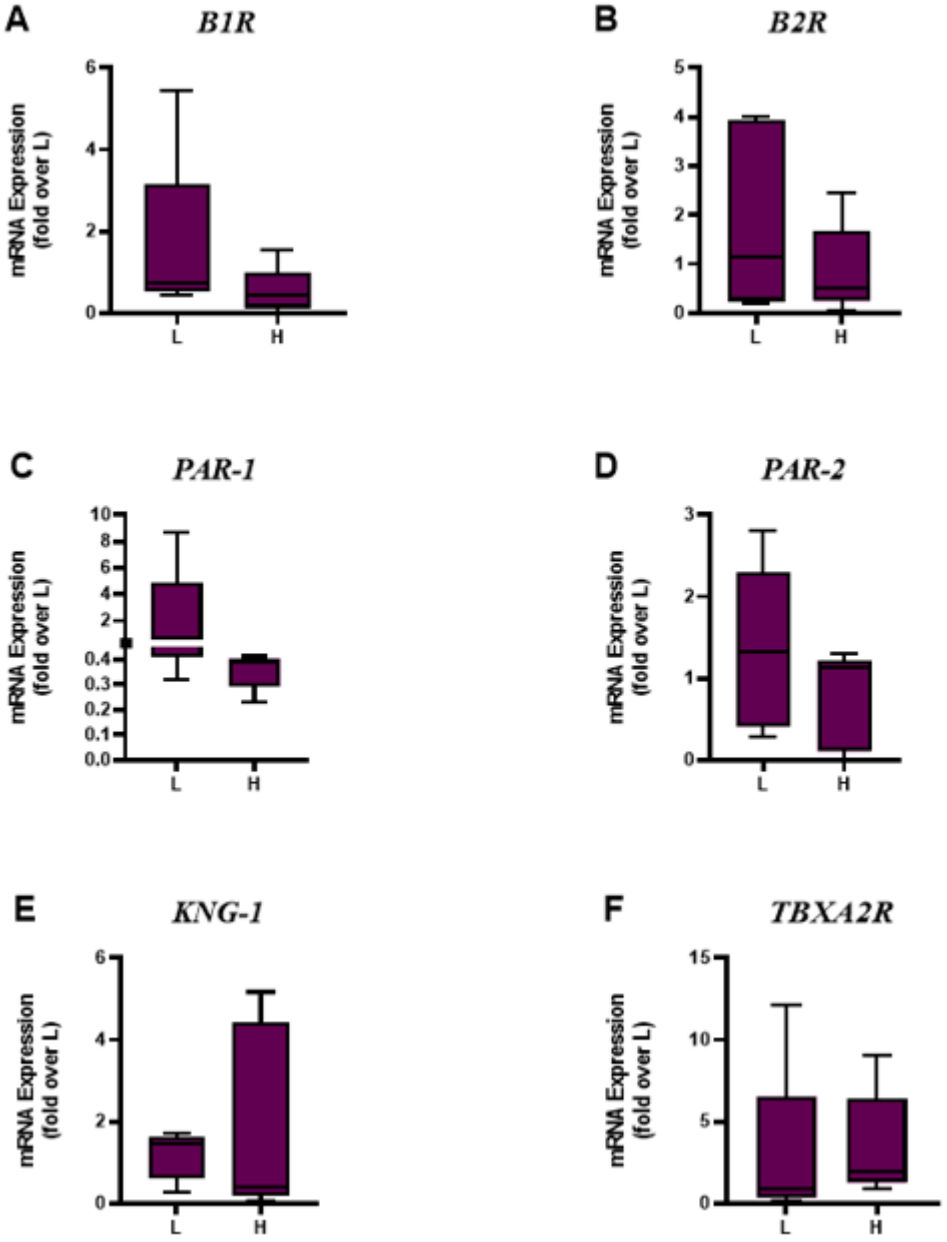

Figure 20. Effect of Hyperglycemia on mRNA Levels of KKS Components in THP-1. mRNA levels of **(A)** B1R, **(B)** B2R, **(C)** PAR-1, **(D)** PAR-2, **(E)** KNG, **(F)** TBXA2R. Data are represented as mean ± SD (N=5). Mann-Whitney U test an Independent t-test were carried out.

2. *The Impact of BK and PK Stimulation on Gene Expression under Normal and Hyperglycemic Conditions:*

a. Inflammatory effect of BK and PK under Normal Glucose Conditions:

Both BK and PK induced the gene expression of IL-6, and Gal-3 compared to unstimulated cells. Moreover, PK alone up-regulated TNF-α gene expression. In contrast, neither BK nor PK increased CTGF gene expression (Fig.21).

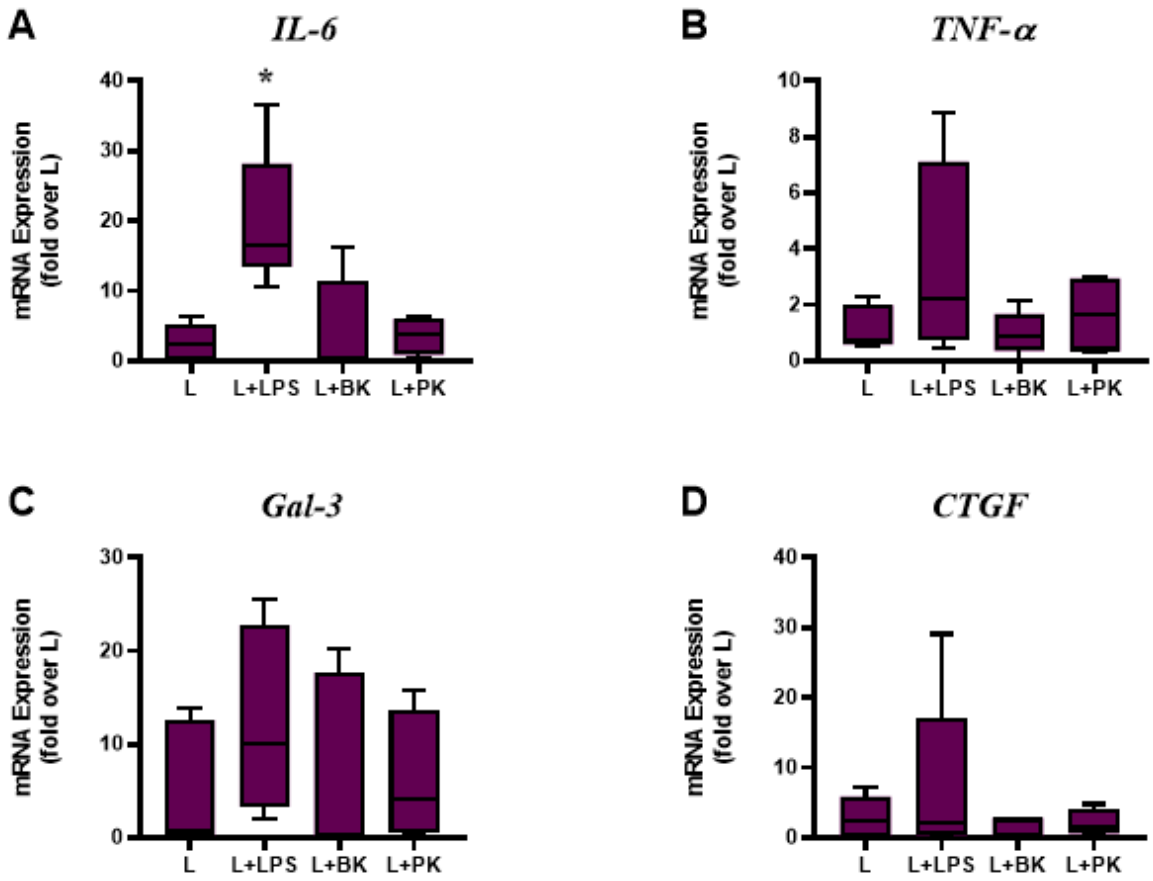

Figure 21.BK and PK Impact on Inflammatory and Fibrotic Gene Expression in Normal Glucose Treated THP-1. The displayed box plots correspond to mRNA levels of **(A)** IL-6 , **(B)** TNF-α, **(C)** Gal-3, **(D)** CTGF. Data are represented as mean ± SD (N= 5), * ($P<0.05$) compared to unstimulated control (L). Mann- Whitney U test was performed.

b. mRNA Levels of KKS Components under Normal Glucose Conditions:

BK and PK promoted the increase of mRNA levels of B2R, KNG, and TBXA2R compared to unstimulated cells. However, no stimulation was observed on B1R, PAR-1, and PAR-2 in response to BK and PK (Fig.22).

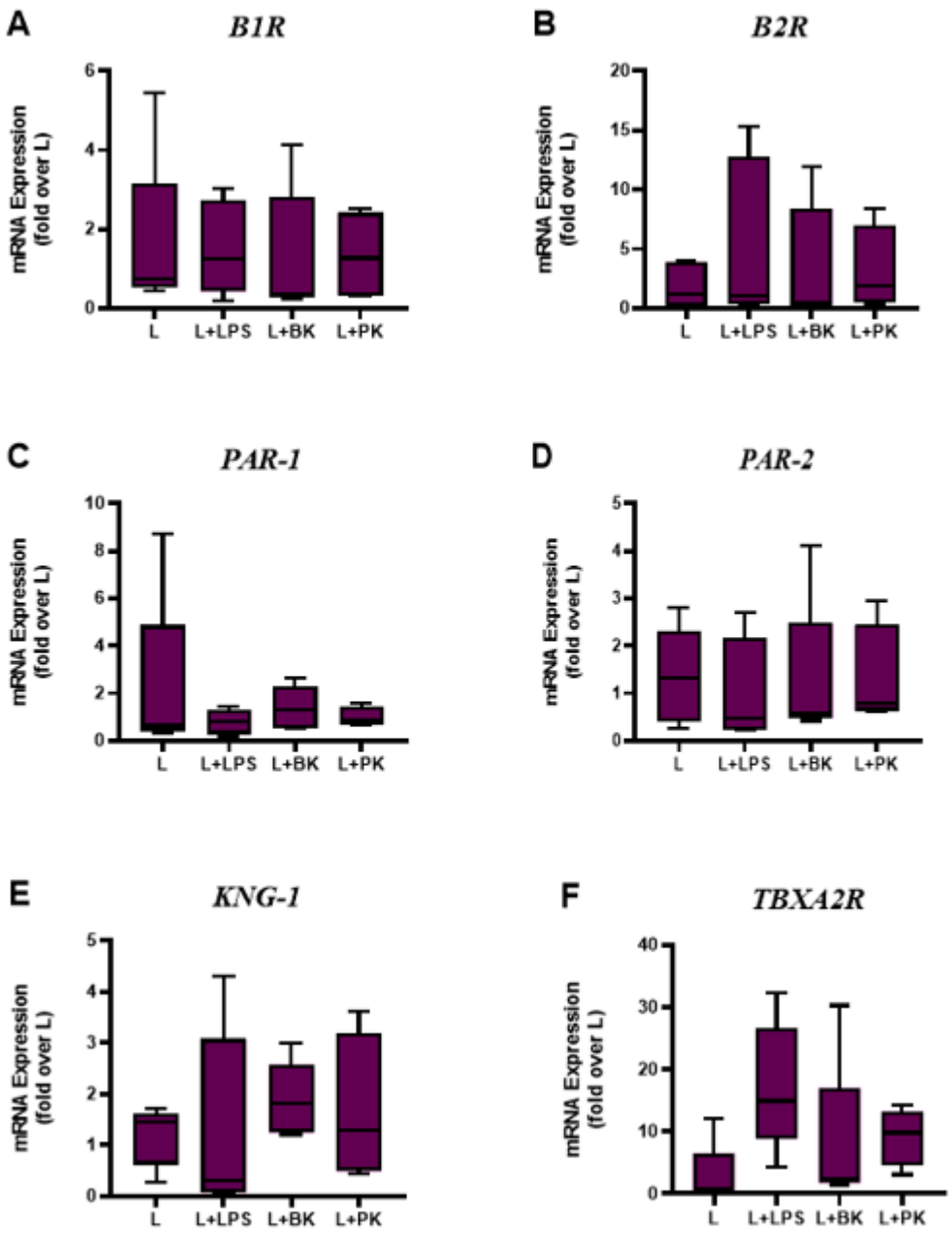

Figure 22. mRNA Levels of KKS Components in Response to BK and PK Stimulation Under Normal Glucose Levels. mRNA levels of **(A)** B1R, **(B)** B2R, **(C)** PAR-1, **(D)** PAR-2, **(E)** KNG, **(F)** TBXA2R. Data are represented as mean ± SD (N= 5). Mann-Whitney U test was performed.

c. Inflammatory Response of THP-1 Macrophages Stimulated with KKS Components under Hyperglycemic Environment:

Under hyperglycemic conditions BK and PK induced the gene expression of IL-6, TNF-α, and CTGF compared to high glucose treated cells without stimulation. Nevertheless, PK inflammatory effect was higher than BK. Furthermore, gal-3 mRNA levels were increased when stimulated with BK but not with PK (Fig.23).

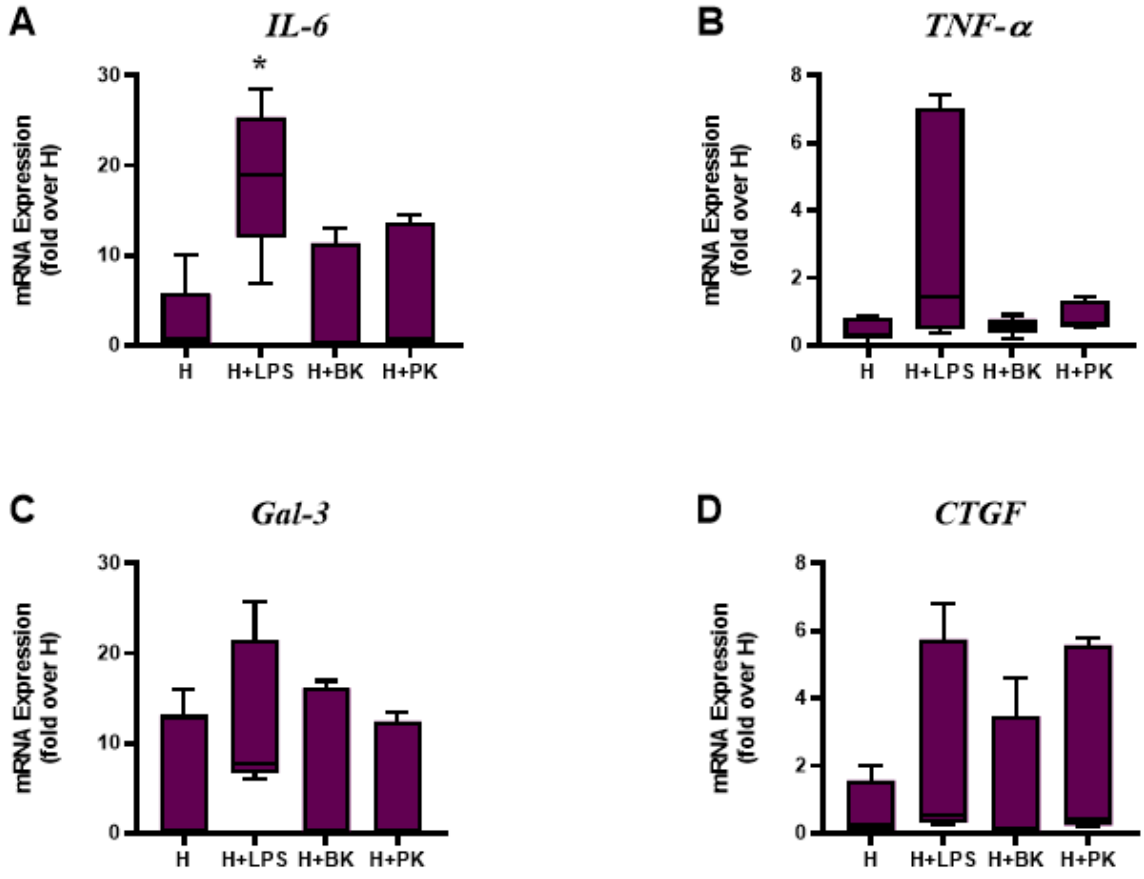

Figure 23. Stimulation with KKS Components in High Glucose Treated THP-1 Cells Enhanced their Pro-inflammatory Response. Gene expression of **(A)** IL-6 , **(B)** TNF-α, **(C)** Gal-3, **(D)** CTGF. Data are represented as mean ± SD (N= 5), * ($P<0.05$) compared to unstimulated control (H). Mann- Whitney U test was performed.

d. mRNA Levels of KKS Components in Response to BK and PK under Hyperglycemic Conditions:

An increase in B2R, PAR-1 and PAR-2 mRNA levels was observed after stimulation with BK and PK compared to unstimulated cells. Also, the mRNA levels of KNG was induced in response to BK stimulation. On the other hand, BK and PK did not stimulate B1R and TBXA2R gene expression (Fig.24).

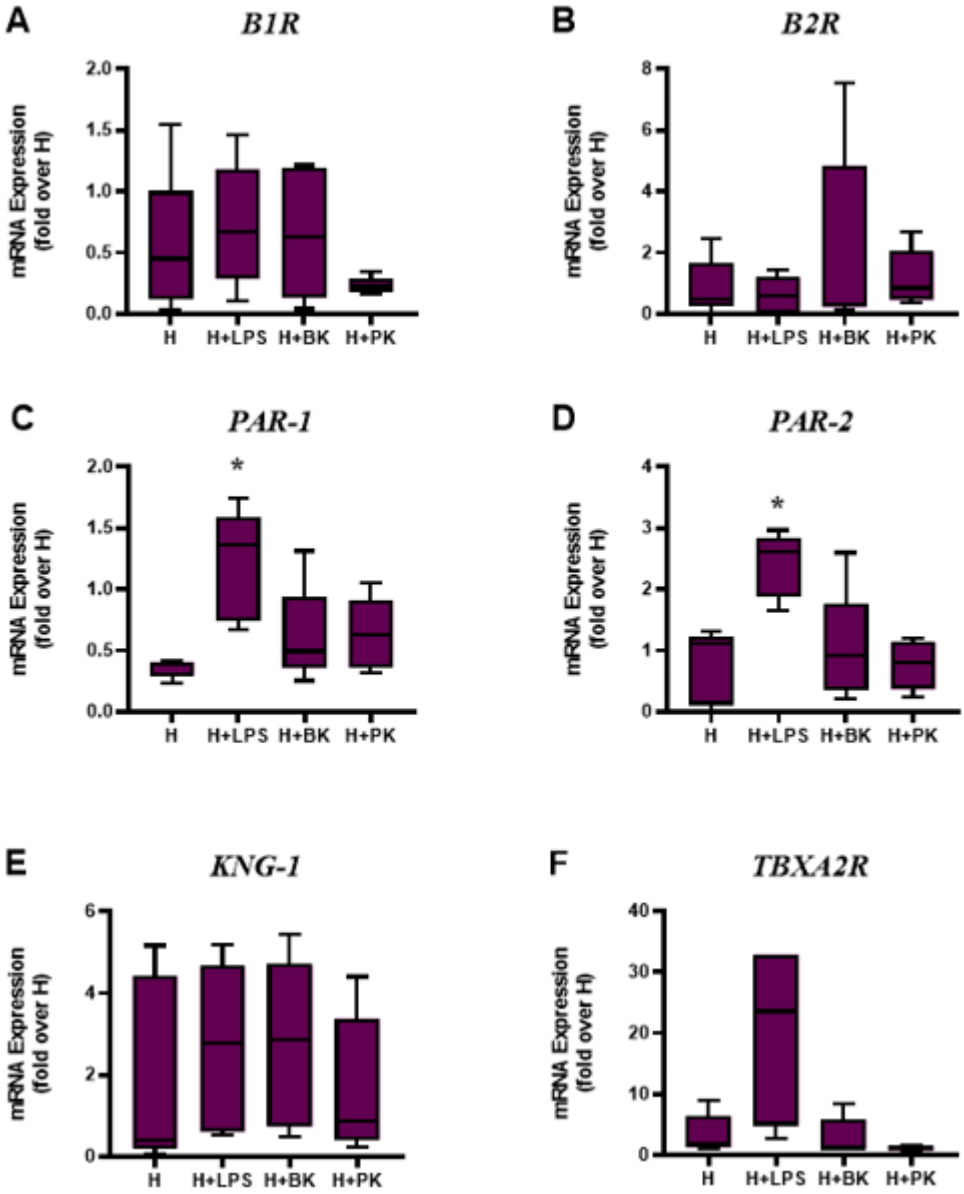

Figure 24. Hyperglycemia in Presence of BK and PK Modulate Gene Expression of KKS Components. mRNA levels of **(A)** B1R, **(B)** B2R, **(C)** PAR-1, **(D)** PAR-2, **(E)** KNG, **(F)** TBXA2R. Data are represented as mean ± SD (N= 5), * ($P<0.05$) compared to unstimulated control (H). Mann- Whitney U test was performed.

D. Measurement of Cytokine Release in THP-1 Macrophages:

1. Hyperglycemic Effect on IL-6 production:

High glucose concentration (20 mM, 14.07± 18.57 pg/ml) did not greatly affect the release of IL-6 compared to normal glucose conditions (5.5mM, 14.15 ± 20.30 pg/ml) (Fig. 25).

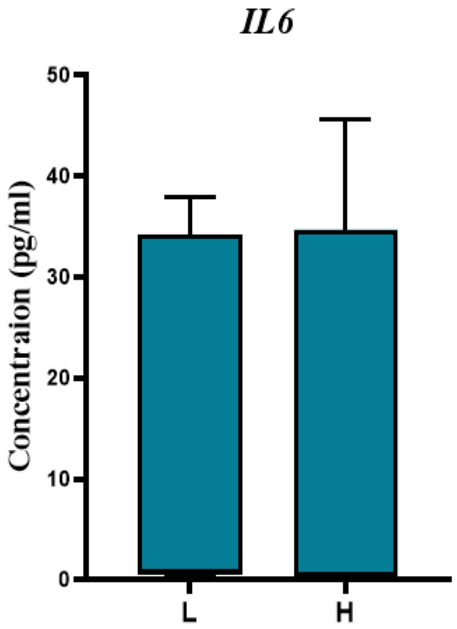

Figure 25: IL-6 Secretion in High Glucose Treated THP-1 Macrophages. Data are represented as mean ± SD (N=5). Mann-Whitney U test was carried out.

2. IL-6 Secretion Following the Stimulation of THP-1 Cells with KKS Components:

a. Effect of BK and PK Stimulation on IL-6 Release under Normal Glucose Conditions:

IL-6 secretion was induced in response to PK. On the other hand, Bk slightly increased IL-6 secretion when compared to unstimulated cells (Fig. 26).

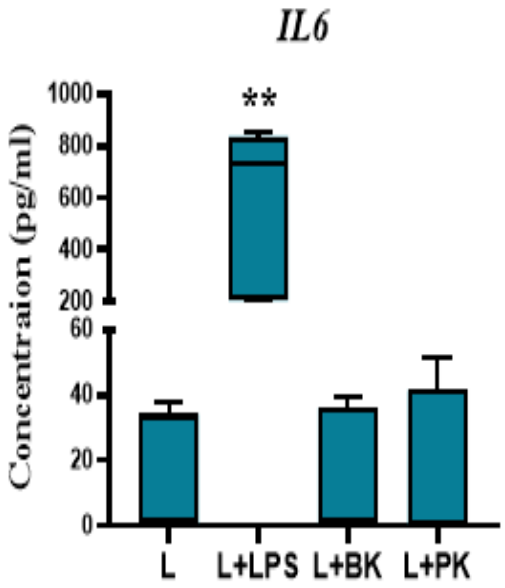

Figure 26. KKS Components Induced Release of IL-6 in THP-1 Cells. Data are represented as mean ± SD (N=5), ** (P<0.01). Mann-Whitney U test was carried out.

b. Effect of BK and PK on IL-6 Release under High Glucose Conditions:

PK stimulated THP-1 cells elevated IL-6 production compared to unstimulated cells in high glucose conditions. Furthermore, BK induced the release of IL-6 but in a lower amount compared to PK (Fig. 27).

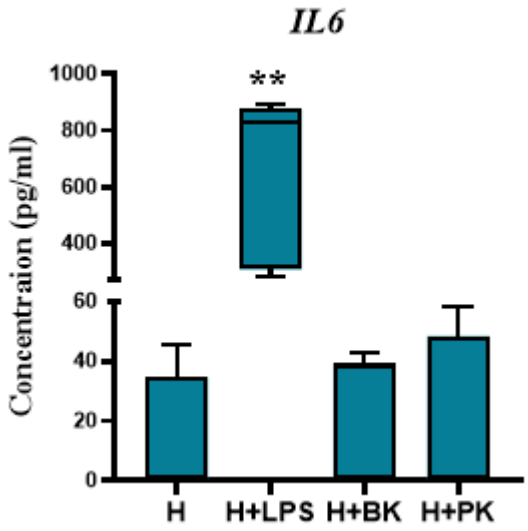

Figure 27. High Glucose in Presence of KKS Components Enhanced IL-6 Production. Data are represented as mean ± SD (N=5), ** (P< 0.001). Mann-Whitney U test was carried out.

E. Determination of PARs Role in Inflammation Induced during Diabetes:

Thp-1 cells were treated with PAR-1 and PAR-2 antagonists followed by stimulation with PK under normal and high glucose levels. Gene expression was validated using RT-qPCR.

1. Effect of PARs Antagonists on Macrophages' Inflammatory Response Under Normal Glucose Conditions:

PAR-1 and PAR-2 antagonists reduced the gene expression of IL-6, Gal-3 and CTGF compared to THP-1 macrophages stimulated with only PK (Fig.28).

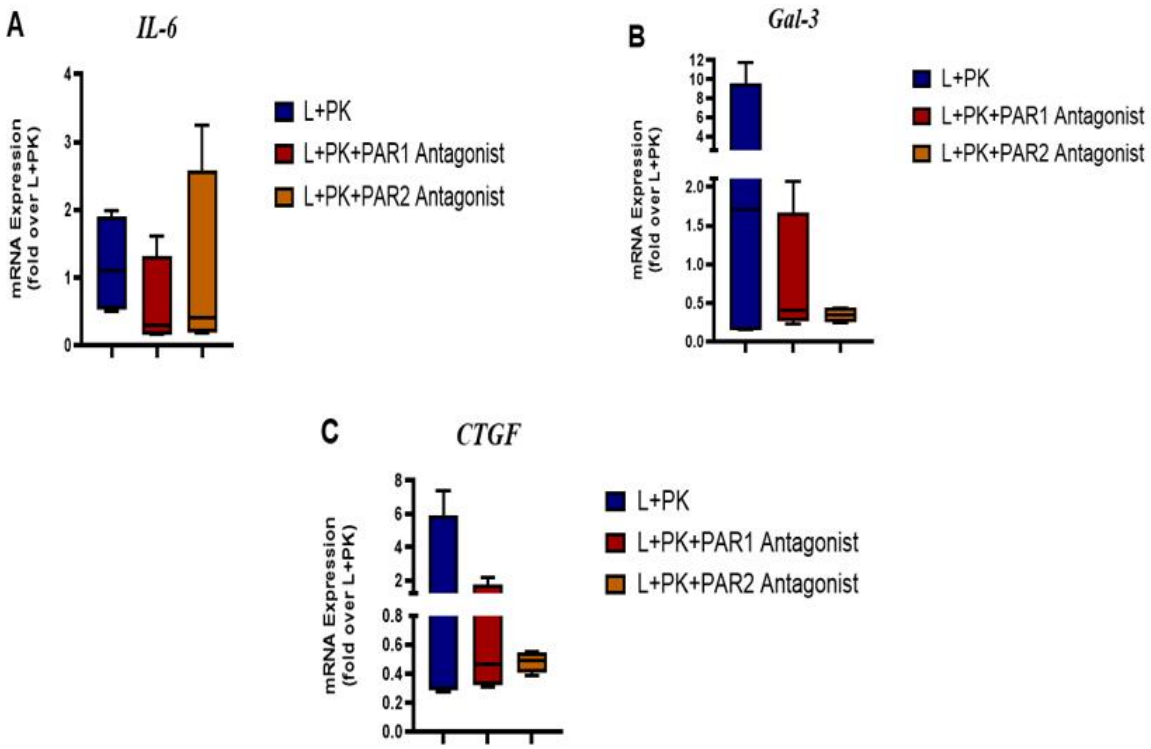

Figure 28: Expression of Inflammatory and Fibrotic Genes Diminished in Response to PARs inhibitors in THP-1 Cells. mRNA levels of **(A)** IL-6, **(B)** Gal-3, **(C)** CTGF. Data are represented as mean ± SD (N= 4). Mann- Whitney U test was performed.

2. *Effect of PARs Antagonists on Macrophages' Inflammatory Response under Hyperglycemic Conditions:*

Gal-3 was reduced after treating macrophages with PAR-1 and PAR-2 antagonists. PAR-1 antagonist was able to decrease IL-6 and CTGF gene expression; however, PAR-2 antagonist had no effect on IL-6 and CTGF mRNA levels (Fig.29).

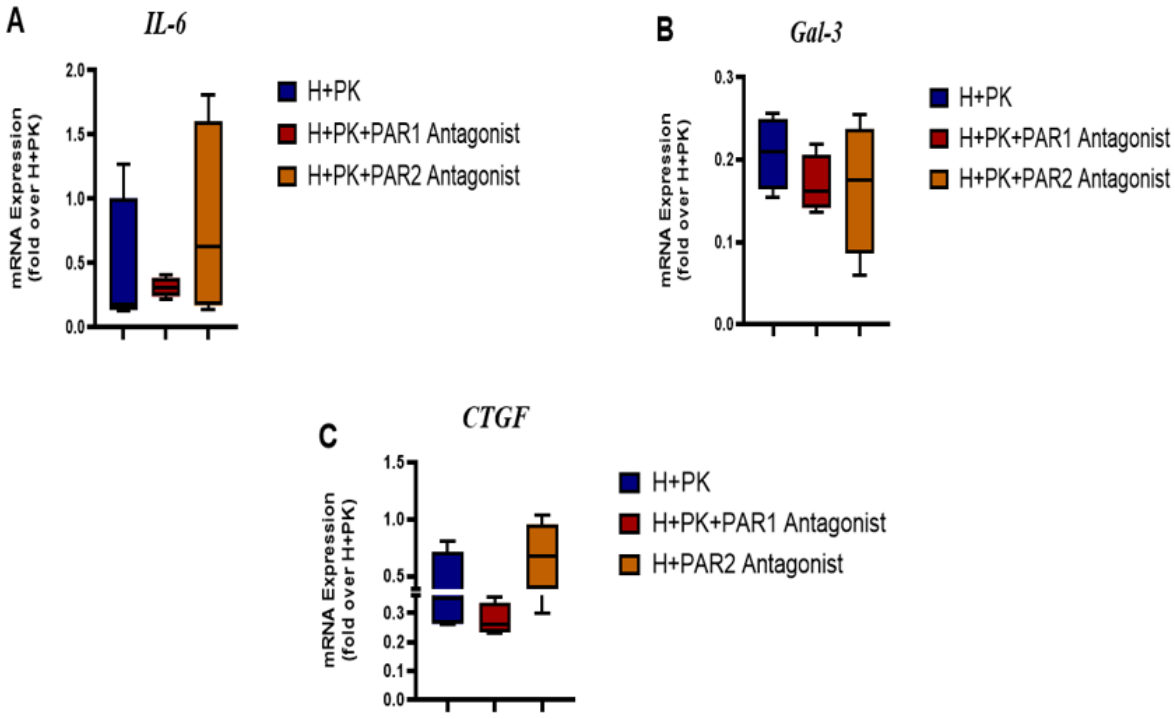

Figure 29. PARs Antagonists under High Glucose Conditions Slightly Inhibited Inflammatory and Fibrotic Gene Expression. mRNA levels of **(A)** IL-6, **(B)** Gal-3, **(C)** CTGF. Data are represented as mean ± SD (N= 4). Mann- Whitney U test was performed.

CHARTER V

DISCUSSION

Emerging evidences suggest that innate immune cells mainly macrophages and their inflammatory processes contribute to the development and progression of chronic diseases such as diabetes. Known that KKS is associated with various physiological processes such as inflammation, vascular permeability, pain, edema, and neovascularization, previous studies indicated that KKS can contribute to micro- and macro-vascular diabetic complications.

In our study we investigated the impact of KKS components (BK and PK) on macrophages' inflammatory response under normal and hyperglycemic conditions. Initially we exposed murine BMDMs and human macrophages (THP-1 cells) to high glucose without any stimulation to assess inflammatory and fibrotic genes. It was found that there was a minor increase mainly in IL-6, Gal, and CTGF. We further observed that high glucose concentration (20 mM) did not affect IL-6 production compared to normal glucose conditions. Then we evaluated the effect of hyperglycemia on mRNA levels of KKS components. And it was shown that hyperglycemia slightly induced the gene expression of TBXA2R and kininogen in both BMDMs and THP-1. However, in high glucose treated BMDMs B1R and PAR2 expression was upregulated. Collectively, this data suggest that hyperglycemia can induce macrophages' pro-inflammatory response and modulate KKS components. However, trying higher glucose concentrations (25-40mM) could have significantly boosted the gene expression of inflammatory, fibrotic and KKS components.

After studying the effect of high glucose on macrophages we stimulated BMDMs and THP-1 cells with BK and PK under normal and high glucose conditions. LPS which is glycoprotein produced by gram negative bacteria and which possess a potent pro-inflammatory action was used as a positive control. The inflammatory effect of both BK and PK was validated in BMDM and THP-1 treated under normal glucose environment through up-regulation of inflammatory and fibrotic gene expression. However, under high glucose PK showed a better pro- inflammatory effect than BK. This propose that high glucose can exacerbate PK inflammatory effect in both BMDMs and THP-1 macrophages. Furthermore, PK and BK promoted macrophages inflammatory response through elevation of IL-6 production under normal and hyperglycemic conditions.

BK and PK stimulated the gene expression of KKS components under normal and high glucose conditions in both types of macrophages. But interestingly in high glucose treated THP-1 macrophages, PK and BK elevated the gene expression of a greater number of KKS components (B1R, B2R, PAR-1, PAR-2 and KNG-1) compared to normal glucose levels and to BMDMs. Thus, KKS components are associated with inflammation induced during diabetes; moreover, BK and PK can mediate their inflammatory effect through bradykinin receptors and PARs. To further confirm these results, we inhibited PARs receptor using PAR1 and PAR2 antagonists in presence of PK in THP-1 macrophages. Both antagonists reduced IL-6, Gal and CTGF gene expression under normal glucose conditions. In contrast, at high glucose the inhibition of inflammatory and fibrotic gene expression was negligible. This suggest that maybe different antagonists' concentration should be used when treating macrophages with PK and high glucose media since as we observed in our results PK inflammatory action increase under high glucose conditions.

In conclusion, our findings demonstrate that KKS components are key regulators of inflammatory processes associated with diabetes. However, in vivo studies should be carried out in order to understand the relation between KKS components and macrophages in diabetes. In addition, it is important to understand the molecular mechanism of KKS in diabetes by focusing on its activated receptors, their crosstalk, and downstream signaling pathways. Overall, our data can provide a promising novel therapeutic approach in treating chronic inflammatory diseases.

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APPENDIX

Table 3: Comparison of BMDMs' Gene Expression in Response to LPS, BK, and PK under Normal Glucose Conditions

Gene Expression	**LPS**	**BK**	**PK**
Increased	IL-6 IL-1β COX-2	Gal-3 CTGF IL-1β B1R B2R, PAR-1 PAR-2 KNG-1 TBXA2R	Gal-3 B1R PAR-2
Decreased	CTGF Gal-3 B1R B2R PAR-1 PAR-2 KNG-1 TBXA2R		KNG-1
Unchanged		IL-6 COX-2	IL-6 COX-2 CTGF IL-1β B2R PAR-1 TBXA2R

Table 4: Comparison of BMDMs' Gene Expression in Response to LPS, BK, and PK under High Glucose Conditions

Gene Expression	**LPS**	**BK**	**PK**
Increased	IL-6 IL-1β COX-2		IL-6 Gal-3 IL-1β CTGF COX-2 B1R B2R
Decreased	CTGF Gal-3 B1R B2R PAR-1 PAR-2 KNG-1 TBXA2R	PAR-2 TBXA2R KNG-1	PAR-1 TBXA2R
Unchanged		CTGF IL-1β IL-6 CTGF Gal-3 B1R B2R PAR-1	PAR-2 KNG-1

Table 5: Comparison of THP-1 Macrophages' Gene Expression in Response to LPS, BK, and PK under Normal Glucose Conditions

Gene Expression	LPS	BK	PK
Increased	IL-6 TNF-α CTGF Gal-3 B2R TBXA2R KNG-1	IL-6 Gal-3 B2R TBXA2R KNG-1	IL-6 TNF-α Gal-3 B2R TBXA2R KNG-1
Decreased	B1R PAR-2	CTGF PAR-1	PAR-1
Unchanged		TNF-α B1R PAR-2	CTGF B1R PAR-2

Table 6: Comparison of THP-1 Macrophages' Gene Expression in Response to LPS, BK, and PK under High Glucose Conditions

Gene Expression	LPS	BK	PK
Increased	IL-6 TNF-α CTGF Gal-3 PAR-1 PAR-2 TBXA2R KNG-1	IL-6 Gal-3 CTGF B2R PAR-1 PAR-2 KNG-1	IL-6 TNF-α CTGF B2R PAR-1
Decreased	B2R		B1R TBXA2R
Unchanged	B1R	TNF-α B1R TBXA2R	Gal-3 PAR-2 KNG-1