REGULATION OF HIGH GLUCOSE INDUCED MONOCYTE CHEMOATTRACTANT PROTEIN –1 GENE IN ENDOTHELIAL CELLS

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REGULATION OF HIGH GLUCOSE INDUCED MONOCYTE CHEMOATTRACTANT PROTEIN –1 GENE IN ENDOTHELIAL CELLS

A thesis presented

by

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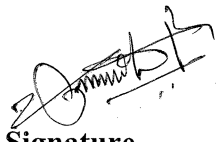
CERTIFICATE

I, **Sumith R Panicker,** hereby certify that I had personally carried out the work depicted in the thesis entitled **"Regulation of High Glucose Induced Monocyte Chemoattractant Protein –1 Gene in Endothelial Cells",** except where external help sought and acknowledged.



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This is to certify that **Mr. Sumith R Panicker**, in the Division of Cellular and Molecular Cardiology of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to his thesis entitled **"REGULATION OF HIGH GLUCOSE INDUCED MONOCYTE CHEMOATTRACTANT PROTEIN –1 GENE IN ENDOTHELIAL CELLS"** was carried out under my direct supervision.

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The thesis entitled

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submitted by

SUMITH R PANICKER

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At the end of writing the thesis acknowledgement page, it always feels good, because hard work on the thesis is left behind. I realize that my research would not be as good as it is now without support and assistance of different people. So, I feel obliged to say thank you one more time.

Finally, I dedicate this thesis to my parents, a constant source of inspiration with unwavering faith in my abilities.

Sumith R Panicker

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ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
AGE	Advanced glycosylated end products
Amp	Ampicillin
AP-1	Activator protein-1
APS	Ammonium persulphate
BAEC	Bovine aortic endothelial cells
BLAST	Basic local search and alignment tool
BSA	Bovine serum albumin
CCR2	CC chemokine receptor 2
cDNA	complementary Deoxy ribonucleic acid
COX-2	Cyclo-oxygenase-2
CPT-1	Carnitine palmitoyltransferase-1
Ct	Threshold cycle
Curc	Curcumin
CVD	Cardiovascular disease
DAB	3,3'-diamino benzidine
DCFH-DA	2',7'–dicholrofluorescein diacetate
DEPC	Diethyl pyrocarbonate
DiI-Ac-LDL	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate labeled Acetylated Low Density Lipoprotein
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide

ECGS	Endothelial cell growth supplement
EDHF	Endothelium derived hyperpolarizing factor
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ET-1	Endothelin-1
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gen	Genistein
HAEC	Human aortic endothelial cell
HASMC	Human aortic smooth muscle cell
HBSS	Hank's balanced salt solution
HG	High glucose
HRP	Horse radish peroxidase
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule
IC_{50}	Half maximal inhibitory concentration
IKK-β	IκB kinase β
IL	Interleukin
iNOS	Inducible NO synthase
IUPAC	International Union of Pure and Applied Chemistry
Kb	Kilobase

KDa	Kilodalton
IκB	I kappa B
LB medium	Luria-Bertani medium
LiCl	Lithium chloride
Los	Losartan
MAP kinase	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MEM	Minimum essential medium
mRNA	messenger Ribonucleic acid
MTT	3-(4-5 dimethyl thiozol-2-yl) 2-5 diphenyl tetrazolium bromide
NAC	N-acetyl cysteine
NaSal	Sodium salicylate
NFκB	Nuclear factor kappa B
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffer saline
PBS-T	Phosphate buffered saline-Tween20
PCAM-1	Platelet cell adhesion molecule-1
PC-PLC	Phosphatidyl choline-phospholipase C
PDT	Population doubling time
PEG	Poly (ethylene glycol)
PGI 2	Prostacyclin

PI 3 kinase	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
Q	Quercetin
RAEC	Rat aortic endothelial cells
RAGE	Receptor for advanced glycosylated end products
RNase	Ribonuclease
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase- polymerase chain reaction
SDS-PAGE	Sodium dodecyl-polyacrylamide gel electrophoresis
TEMED	N, N, N', N' - tetramethylethylenediamine
TNF	Tumour necrosis factor
TPA	Tetradecanoylphorbol Acetate
t-PA	tissue-Plasminogen activator
TRITC	Texas red isothiocyanate
TXA2	Thromboxane A2
VCAM-1	Vascular cell adhesion molecule-1
VSMC	Vascular smooth muscle cell
h	Hour
s	Seconds
µg	Microgram
µl	Microlitre
µM	Micromolar

SYNOPSIS

Cardiovascular complications are the leading cause of morbidity & mortality in patients with type 2 diabetes. The onset and progression of vascular complications such as atherosclerosis are delayed in patients with good glycemic control. Hence hyperglycemia is thought to be an important regulator of vascular lesion development. Hyperglycemia results in of the endothelium dysfunction and hyperactivity of vascular smooth muscle cells and platelets etc. which in turn results in oxidative stress, increased polyol pathway and advanced glycosylated end products (AGE) formation, activation of protein kinase C (PKC) and receptor for AGE (RAGE). All of these contribute to vascular complications associated with type 2 diabetes. But the underlying mechanisms between hyperglycemia and accelerated rate of atherosclerosis are not fully clarified.

The following questions were raised: (i) Does high ambient glucose (HG) induce aortic endothelial cells to synthesize monocyte chemoattractant protein -1 (MCP-1)? (ii) Whether transcription factors such as nuclear factor kappa B (NFκB) and activator protein-1 (AP-1) regulate MCP-1 gene expression in endothelial cells under high glucose condition?

It was hypothesized that MCP-1 may get preferentially activated in aortic endothelial cells exposed to high ambient glucose and that high levels of glucose may induce MCP-1 gene expression *via* activation of transcription factors such as NFκB and AP-1 through various signaling pathways. The present study is aimed at elucidating molecular mechanisms involved in high ambient glucose induced MCP-1 gene expression in aortic endothelial cells.

MCP-1 is a CC Chemokine, which plays an important role in the adhesion and migration of monocytes into the arterial intima, an early critical event in atherogenesis. It is also involved in other cardiovascular disease pathogenesis such as acute coronary syndrome, graft vasculopathy and restenosis, vascular remodeling owing to hypertension, myocarditis / cardiomyopathy, cardiac dysfunction and remodeling after MI. Both MCP-1 and the adhesion molecules are crucial in atherogenesis. Evidences suggest MCP-1 and its leukocyte receptors, the CC chemokine receptors (CCR2) to play a key role in initiation of atherosclerosis with endothelial cells and subendothelial macrophages to be the major sources of MCP-1 in early atherosclerotic lesions. In earlier studies, abrogation of MCP-1/ CCR2 pathway has been found to inhibit the early development of atherosclerotic lesions in mice and the deletion of MCP-1 gene in transgenic mice expressing human *apo*B showed a dramatic decrease in macrophage recruitment and atherosclerotic lesion formation. Recently anti–MCP-1 gene therapy was found to attenuate the progression of established atherosclerosis in Apo lipoprotein E-knockout mice. These findings suggest that MCP-1 contributes to the initiation and development of atherosclerosis. Studies have reported increased expression of MCP-1 in type 2 diabetes and suggested that increased MCP-1 expression may contribute to the increased risk for atherosclerosis in type 2 diabetes. But role of MCP-1 in the increased rate of atherosclerosis in type 2 diabetes remains to be elucidated. Molecular mechanisms involved in the transcriptional and translational regulation of MCP-1 gene expression in aortic endothelial cells in response to HG remains also to be explored. Two NFκB, one AP-1 and one Sp-1 binding sites have been characterized upstream of MCP-1 gene in humans, mice and rats. Induction of MCP-1 gene expression is strongly dependent on

activation of transcription factor NFκB whereas basal transcription is regulated by Sp-1. Certain key signalling pathways involved in the activation of MCP-1 gene such as PKC, p38 MAP kinase and oxidative stress induced by high levels of glucose have been shown to up regulate MCP-1 gene expression *via* activation of NFκB. Even though several studies have shown transcription factors such as AP-1, Sp-1, Smad and Peroxisome Proliferator-Activated Receptors (PPARs) to be involved in MCP-1 transcriptional regulation under different stimuli, not much is known regarding their role in regulating MCP-1 gene expression in endothelial cells under high glucose conditions. Further, no studies have been done to elucidate the possible interaction among different transcription factors such as AP-1, Sp-1 and NFκB leading to altered MCP-1 expression during high glucose conditions. The subunit composition of transcription factors namely NFκB and AP-1 involved in high glucose induced MCP-1 transcription remains to be elucidated. Data is also not available regarding the MCP-1 mRNA and protein synthesis in a dose and time dependant.

Present studies were undertaken to fill some of the above lacunae. As the differential induction and binding of transcription factors to the promoter region of the MCP-1 gene are critical regulatory steps, analyzing MCP-1 gene expression in specific cell types and under specific stimulus may be expected to delineate the transcriptional control of MCP-1 gene in distinct pathological conditions and serve to identify specific targets for intervention.

Given the above background, the role of NFκB and AP-1 in regulating the expression of MCP-1 in rat aortic endothelial cells (RAECs) under HG conditions was explored.

Further, the modulating effect of curcumin, quercetin, genistein, losartan and sodium salicylate on HG induced MCP-1 expression in RAECs was also studied. These agents were chosen because they are already reported to have anti-inflammatory, anti-thrombotic and antioxidant activity and considered to abate high glucose induced MCP-1 expression in aortic endothelial cells. Molecules showing attenuation of HG induced MCP-1 expression were studied for the molecular basis for their inhibitory action.

The entire studies were done on aortic endothelial cells isolated from male Sprague-Dawley rats (**RAECs**) except for transfection studies where **EA.hy 926** cells were used. The isolated RAECs were characterized by their cobblestone morphology and by their uptake of **1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate- Acetylated LDL** . The cells were also characterized by positive staining for vWF and vimentin. Glucose concentration of 5.5 mM was taken as control and 15, 25 and 35 mM were taken as HG concentrations. The cells when exposed to different HG glucose concentrations showed increased MCP-1 protein secretion in a dose dependent manner as determined by **enzyme linked immuno sorbent assay**. The viability of RAECs exposed to HG concentrations of 35 mM was found to be significantly affected as determined using **[3-(4-5 dimethyl thiozol-2-yl) 2-5 diphenyl tetrazolium bromide assay**. Hence 25mM was chosen as the HG for the remaining studies. The MCP-1 mRNA levels in RAECs exposed to different treatment conditions were determined using **semi-quantitative** and **Real-time Reverse Transcriptase-Polymerase Chain Reaction analysis**. D-mannitol concentration of 19.5mM did not induce MCP-1 protein and mRNA synthesis ruling out the possibility that osmolality as the reason for HG induced

increased MCP-1 synthesis. Upon HG stimulation there was increased NFκB and AP-1 DNA binding activity as determined using **electrophoretic mobility shift assay** and the subunit composition determined by **supershift analysis** showed that NFκB bands are composed of p50-p65 heterodimer whereas AP-1 bands were composed of c-jun, c-fos and fra-1 subunits. As increased promoter activity is essential for increased gene transcription **dual luciferase reporter assay** was done in **EA.hy 926 cell line** exposed to HG using pMCP-1 Luc containing the MCP-1 gene promoter corresponding to nucleotides -2466 to +67 cloned into pGL3 basic luciferase vector. The MCP-1 gene promoter activity was significantly increased upon HG stimulation in EA.hy 926 cells. Similarly dual luciferase reporter assay was also done with **pNFκB Luc** containing NFκB binding sequence (-2407 to -2065) in the MCP-1 promoter **cloned** into pGL3 promoter vector and **pAP-1 Luc** containing AP-1 binding sequence (-241 to -95) in the MCP-1 promoter **cloned** into pGL3 promoter vector. It was found that NFκB and AP-1 enhancer activity was also increased in EA.hy 926 cells exposed to HG. Luciferase activity was normalized with pRL-TK.

The different molecules such as curcumin (15 μM , 30 μM) and quercetin (100 μM) screened for their modulating effect on HG induced MCP-1 expression significantly attenuated HG induced MCP-1 protein and mRNA synthesis. Losartan, sodium salicylate and genistein did not attenuate HG induced MCP-1 synthesis. Therefore, the molecular mechanism by which curcumin and quercetin could possibly attenuate HG induced MCP-1 expression was further explored. Both curcumin (30 μM) and the specific inhibitor of NFκB, Bay 11-7082 (5 μM) significantly attenuated HG induced MCP-1 mRNA

synthesis. Curcumin and Bay 1-7082 also decreased NFκB DNA binding activity. This suggests that NFκB is involved in HG induced MCP-1 gene expression and curcumin attenuates HG induced MCP-1 expression partly via NFκB.

Molecular mechanism by which quercetin inhibited HG induced MCP-1 gene expression in aortic endothelial cells was explored. Quercetin was found to significantly attenuate HG induced MCP-1 mRNA synthesis almost to the control level. The antioxidant N-acetyl cysteine however did not bring down HG induced MCP-1 mRNA levels to the control levels even though MCP-1 mRNA levels were significantly decreased compared to the HG treated group. Quercetin as expected was able to prevent ROS generation as detected by **DCFH-DA staining**. In **western blot analysis** it was seen that there was increased nuclear translocation of the cytoplasmic p65 upon HG induction and quercetin almost completely retained p65 in the cytoplasm similar to that of control. Whereas N-actyl cysteine retained p65 only partially when compared with control. An increased p65 translocation into the nucleus upon HG stimulation was seen by **confocal imaging** and quercetin could prevent the p65 translocation. These findings support the result obtained by western blotting for cytopolasmic p65 translocation. Similar to the action of curcumin, quercetin also inhibited HG induced NFκB DNA binding activity. Quercetin prevented HG induced AP-1 DNA binding activity. In Dual luciferase reporter assay, quercetin significantly inhibited MCP-1 gene promoter as well as NFκB and AP-1 activation upon HG stimulation. These results suggest that HG induces MCP-1 synthesis in RAECs through NFκB and AP-1 and quercetin attenuates HG induced MCP-1 gene expression mainly by dual inhibition of NFkB and AP-1 activation.

Major Findings

1. High glucose induces MCP-1 protein and mRNA synthesis in RAECs in a dose and time dependent manner and this increased synthesis of MCP-1 is not due to osmolality.
2. High glucose induces ROS generation, increased p65 nuclear translocation and MCP-1 gene promoter activation.
3. High glucose increases NFκB and AP-1 DNA binding activity as well as NFκB and AP-1 enhancer activation.
4. The upregulation of MCP-1 gene is primarily mediated by transcription factors NFκB and AP-1.
5. ROS is only partly responsible for the HG induced MCP-1 gene expression in aortic endothelial cells.
6. Curcumin attenuates HG induced MCP-1 expression partly by inhibiting NFκB.
7. Quercetin attenuates HG induced MCP-1 gene expression and the mechanism involves inhibiting ROS generation, preventing NFκB and AP-1 DNA binding activity as well as activation of MCP-1 gene promoter.

Significance of the current findings

1. The increased synthesis of MCP-1 by aortic endothelial cells exposed to HG may partly explain the increased risk for atherosclerosis in patients with type 2 diabetes.
2. The finding that quercetin and curcumin inhibits HG induced MCP-1 synthesis suggests that both the above molecules have the potential to be developed as a drug for abating accelerated rate of atherosclerosis in type 2 diabetes.

I. INTRODUCTION

I.A.1.Atherosclerosis and type 2 diabetes

Atherosclerotic cardiovascular diseases and the most common form of diabetes namely type 2 diabetes are significant health problems worldwide. Type 2 diabetes mellitus is a powerful and independent risk factor for several forms of cardiovascular diseases in both men and women (e.g. coronary artery disease, stroke, peripheral arterial disease, cardiomyopathy and congestive heart failure). Additionally, cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes. One interesting fact is the accelerated rate of progression of atherosclerosis in patients with type 2 diabetes. The reasons for the above are not clear.

The pathophysiology of vascular disease in diabetes involves abnormalities in endothelial, vascular smooth muscle cell and platelet functions. The metabolic abnormalities that characterize diabetes such as hyperglycemia, increased fatty acids and insulin resistance contribute to molecular mechanisms that alter the structure and function of blood vessels [1].

I.A.2. Hyperglycemia and endothelial dysfunction in type 2 diabetes

Hyperglycemia is considered to be one of the important regulators of vascular lesion development in type 2 diabetes [2, 3]. Patients with good glycemic control are found have retarded rate of atherosclerosis. The underlying mechanisms between hyperglycemia and accelerated rate of atherosclerosis in type 2 diabetes are not completely understood. Prolonged exposure to hyperglycemia leads to increased oxidative stress, disturbances in intracellular signal transduction such as activation of PKC, p38MAP kinase etc., non-enzymatic glycosylation of proteins and lipids leading to AGE formation, activation of

RAGE, all of which lead to activation or dysfunction of endothelial cells [4]. Endothelial dysfunction is thus a key feature of diabetes mellitus and is thought to be the major cause of vascular complications associated with the disease. Endothelial dysfunction is seen even before the development of clinically manifested vascular disease. Endothelial dysfunction results in decreased nitric oxide and PGI 2 production and increased ET-1 and vasoconstrictor prostanoids promoting vasoconstriction. Activation of transcription factors such as NFκB, AP-1 etc. leading to an upregulation of chemokines (MCP-1, IL-8 etc.), cytokines (IL-1, IL-6, IL-10 etc.) and cell adhesion molecules (Selectins, ICAM-1, VCAM-1 etc) promote inflammation whereas upregulation of vWF, tissue factors and PAI-1 promote coagulation and thrombosis. Increased growth factor expression leads to vascular smooth muscle proliferation. All these alterations resulting from endothelial dysfunction may contribute to increased risk for atherosclerosis [5, 6].

I.A.3. Monocyte Chemoattractant Protein -1 (MCP-1)

One of the primary mechanisms by which hyperglycemia induced endothelial dysfunction may cause accelerated rate of atherosclerosis in type 2 diabetes is by the increased production of MCP-1. MCP-1 is a chemoattractant member of the chemokine superfamily implicated in pathogenesis of atherosclerosis, inflammatory responses, immune regulation, wound healing, tissue remodeling and modulation of tumor behaviour. The action of MCP-1 is thought to be related to its chemotactic effects. This chemokine is responsible for monocyte recruitment in acute and chronic inflammatory states. Both MCP-1 and the adhesion molecules are crucial in atherogenesis. MCP-1 and its receptor CCR2 are recognized to have an important role in the adhesion and migration

of monocytes into the arterial intima, an early critical event in atherogenesis. MCP-1 is involved both in the initiation and progression of atherosclerosis. MCP-1 expression by endothelial cells contributes to the establishment of a chemokine gradient that facilitates subset specific recruitment of leukocytes to the sites of inflammatory challenge. Under flow conditions, MCP-1 triggers firm adhesion of rolling monocytes to E-Selectin-expressing vascular endothelium, thus contributing to recruitment of monocytes from the vascular compartment [7]. At least three independent pathways regulate MCP-1 gene expression in activated endothelial cells. Its induction requires activation of IKK-β / IκB alpha /NFκB signalling pathway resulting in nuclear accumulation of p65 and subsequent recruitment of cofactors. Proper assembly and activity of this transcriptional complex is further modulated by p38 MAP kinase cascade and PC-PLC.

I.A.4. MCP-1 regulation in high glucose conditions

The molecular mechanism leading to MCP-1 gene expression in endothelial cells during hyperglycemic conditions in Type 2 diabetes mellitus is yet to be elucidated. The transcriptional and translational regulation of MCP-1 gene have not been characterized in high glucose (HG) stimulated endothelial cells. Patients with diabetes have increased levels of serum MCP-1. In monocytes and endothelial cells exposed to HG there is increased MCP-1 mRNA and protein synthesis. Given that high glucose accelerates MCP-1 synthesis in vascular endothelial cells, it is reasonable to speculate that MCP-1 contributes to the initiation and progression of vascular lesions in type 2 diabetes.

The induction of MCP-1 gene expression is strongly dependent on activation of transcription factor NF-κB whereas basal transcription is regulated by Sp-1. Under HG

conditions, certain key signaling pathways such as Protien kinase C, p38 MAP kinase and ROS have been shown to upregulate MCP-1 gene expression *via* activation of NFκB. Data is not available whether HG induces the MCP-1 mRNA and protein synthesis in a dose and time dependant manner. Though several studies have shown transcription factors such as AP-1, Sp-1, Smad and PPARs to be involved in MCP-1 transcriptional regulation under different stimuli, not much is known regarding their role in regulating HG induced MCP-1 gene expression in endothelial cells. Further, no studies have been done to elucidate the possible interaction among different transcription factors such as PPARs, Smad, AP-1, Sp-1 and NFκB leading to altered MCP-1 gene expression upon HG stimulation. The subunit composition of transcription factors namely NF-κB and AP-1 involved in HG induced MCP-1 transcription have not yet been elucidated.

The studies for my PhD programme were envisaged to fill some of these lacunae. The differences in the induction and binding of transcription factors to the MCP-1 gene promoter is a critical regulatory step that causes the MCP-1 gene to express in a specific cell and in a stimulus specific manner. Delineating the transcriptional control of MCP-1 gene may help us to identify specific targets to reduce the risk for atherosclerosis in type 2 Diabetes.

I.B. AIMS:

(i) To find the mechanism of regulation of MCP-1 gene in aortic endothelial cells exposed to high glucose concentrations and (ii) To determine whether agents such as quercetin, curcumin, losartan, sodium salicylate and genistein known to have anti-inflammatory, anti-thrombotic or antioxidant activity would abate high ambient glucose induced MCP-1 expression in aortic endothelial cells.

The larger goal is identification of agents that can possibly reduce the risk of atherosclerosis in diabetes mellitus.

I.C. OBJECTIVES:

- To standardize the isolation and culture of aortic endothelial cells from rat.
- To determine the population doubling time and viability of rat aortic endothelial cells exposed to different glucose concentrations.
- To determine whether high concentrations of glucose up regulates MCP-1 gene expression in aortic endothelial cells in a dose and time dependent manner.
- To determine the role of reactive oxygen species in high glucose induced MCP-1 gene expression in aortic endothelial cells.
- To delineate the specific role of NFκB, and AP-1 in the transcriptional induction of MCP-1 gene in aortic endothelial cells.
- To characterize the subunit composition of NFκB and AP-1 involved in MCP-1 gene expression in endothelial cells under high glucose conditions.
- To observe whether high glucose induced expression of MCP-1 by rat aortic endothelial cells can be attenuated by various molecules such as quercetin, genistein, sodium salicylate, losartan and curcumin and study the mechanism of their action.

I.D. HYPOTHESIS

We hypothesized that 'In RAECs exposed to higher glucose concentrations, MCP-1 may get preferentially activated and that HG induces MCP-1 gene expression *via* activation of transcription factors such as NFκB and AP-1 through various signaling pathways. Any pharmacological inhibitor that can interfere with the activation of these transcription factor may be expected to modulate HG induced MCP-1 expression'.

II. REVIEW OF LITERATURE

II.1. ATHEROSCLEROSIS

Atherosclerosis has been affecting humans since 3000 BC. Extensive macroscopic and microscopic evidences for atherosclerosis have been found in the aortaeand carotid arteries of mummies. Vascular lesions in mummies have similar pathologic features that are observed in humans in modern times [8]. The term 'atherosclerosis' comes from the Greek words '*athero*' meaning gruel or paste and '*skleros*' meaning hard. According to Russell Ross, Professor of Pathology at University of Washington School of Medicine in Seattle, 'atherosclerosis' is not merely a disease in its own right but a process that is the principal contributor to the pathogenesis of myocardial and cerebral infarction, gangrene and loss of function in the extremities. Atherosclerosis is a chronic and insidious disorder. The early atherosclerotic lesions, present even in teenagers and young adults, evolve over decades before manifesting clinically [9].

Atherosclerosis is a disease of the arterial wall that occurs at susceptible sites in the major conduit arteries and constitutes one of the most common medical and surgical problems manifesting clinically as coronary artery disease (CAD), peripheral vascular disease (PVD) and stroke. Lesions begin in the inner lining of the arteries—the intima—and they progressively affect the entire arterial wall, including the media and the adventitia **(Figure 1)** [10]. The hallmark of the disease is the fibrofatty plaque and its evolution involves endothelial injury, lipid retention, oxidation, and modification, which initiates chronic inflammation in the arterial wall. Atherosclerotic lesions can cause stenosis with

potentially lethal distal ischemia or can trigger thrombotic occlusion of major conduit arteries to the heart, brain, legs, and other organs.

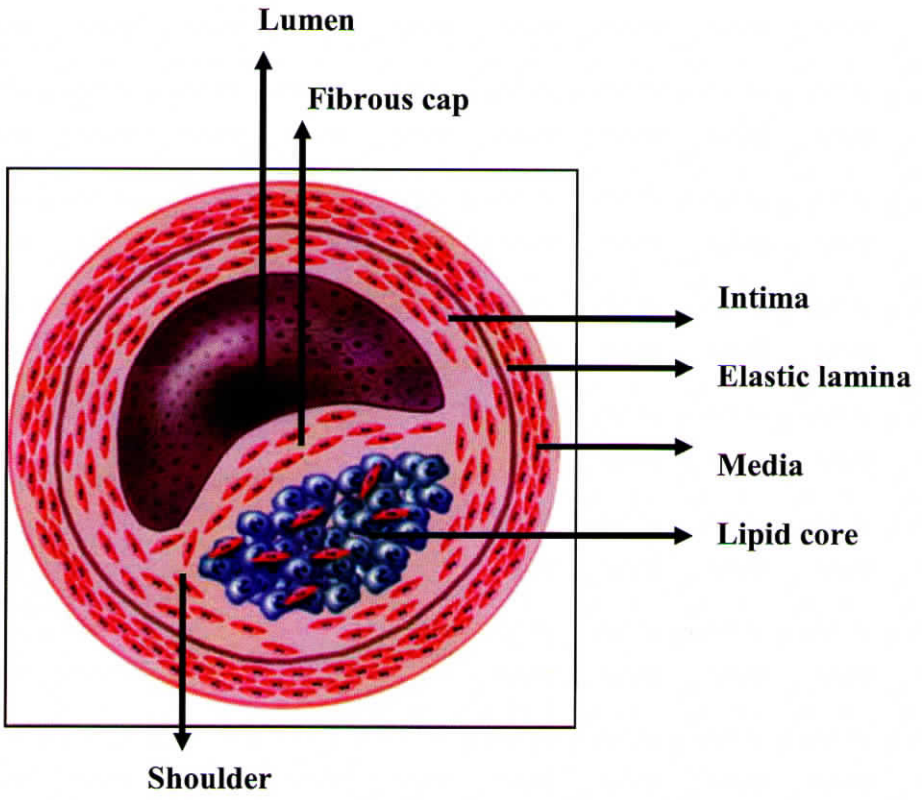

Figure 1: Schematic representation of an atheroma (type IV lesion). An eccentric lesion with lipid pool and fibrous cap is seen. (Braunwald's Heart Disease, 8th edition)

II.2. THEORIES ON ATHEROSCLEROSIS

Several theories have been proposed to explain the initiation and progression of atherosclerotic plaques. All these theories are not mutually exclusive, and are indeed linked to each other.

The first written description of atherosclerosis is by Joseph Conrad Bruner who, in 1695, described `hardening' of the aorta and major blood vessels seen at an autopsy.

II.2.1. Thrombogenic or encrustation theory

In 1852, Carl von Rokitansky postulated the 'thrombogenic' or 'encrustation' theory, which stated that atherosclerosis begins in the intima with deposition of thrombus, leading to organization by infiltration of fibroblasts and secondary lipid deposition, associated with some form of injury to the artery.

II.2.2. Inflammatory theory

In 1856, Rudolph Virchow proposed the 'inflammatory' theory which states that lesions of atherosclerosis results from some form injury to the arterial wall with lipid transudation into the arterial wall and its interaction with cellular and extracellular elements causing intimal proliferation.

Both these theories took into account the accumulation of lipid as a primary or secondary phenomenon in association with arterial injury.

II.2.3. Lipid theory

In the early 1900s A Ignatowsky, S Saltykow and NN Anitschkow first put forward the 'lipid' theory of atherogenesis, and described the importance of the relationship between circulating cholesterol levels and atherosclerosis [11].

In 1946, Duguid proposed that intimal thickening occurred as a result of the accumulation of fibrin and platelets. In 1966, JE French stressed the inflammatory components as important in the development of lesions of atherosclerosis. Wissler and colleagues showed that smooth muscle cell accumulation or proliferation was a key element in the development of lesions of atherosclerosis.

II.2.4. Hemodynamic theory

The 'hemodynamic' theory, first proposed in 1950, attempted to explain the focal nature of atherosclerosis and suggested that hydrostatic and shear forces are mainly responsible for the development of the lesions. Evidence in support is the fact that hypertension predisposes to the development of atheroma and that the lesions have a predilection for the branching sites of the arterial system where turbulent or relatively stagnant flow with oscillating or low shear stress is usually detected [12, 13].

II.2.5. Response to injury hypothesis

In 1973, Russell Ross along with John Glomset, formulated the "response to injury" hypothesis of atherosclerosis. This is a more unifying theory that postulates that "atherosclerosis starts with endothelial injury making the endothelium susceptible to the accumulation of lipids and the deposition of thrombus". This hypothesis has been tested and modified from its original formulation. The response to injury hypothesis has had a profound impact on atherosclerosis research and vascular biology and is still the most widely accepted theory on atherosclerosis. The lipid, thrombogenic and inflammatory theories have subsequently been combined into our modern concept of atherogenesis.

II.3. STRUCTURE OF A NORMAL ARTERY (Figure 2)

A large artery consists of three morphologically distinct layers- the intima, the media and the adventitia.

A. The **intima** is the inner most layer bound by a monolayer of endothelial cells on the luminal side and an abluminal sheet of elastic fibres (the internal elastic lamina). The sub intima is very thin and consists of extracellular connective tissue such as proteoglycans and collagen.

B. The **media** is the middle layer consisiting of vascular smooth muscle cells.

C. The **adventitia** is the outermost layer consisting of connective tissues interspersed with fibroblast and vascular smooth muscle cells.

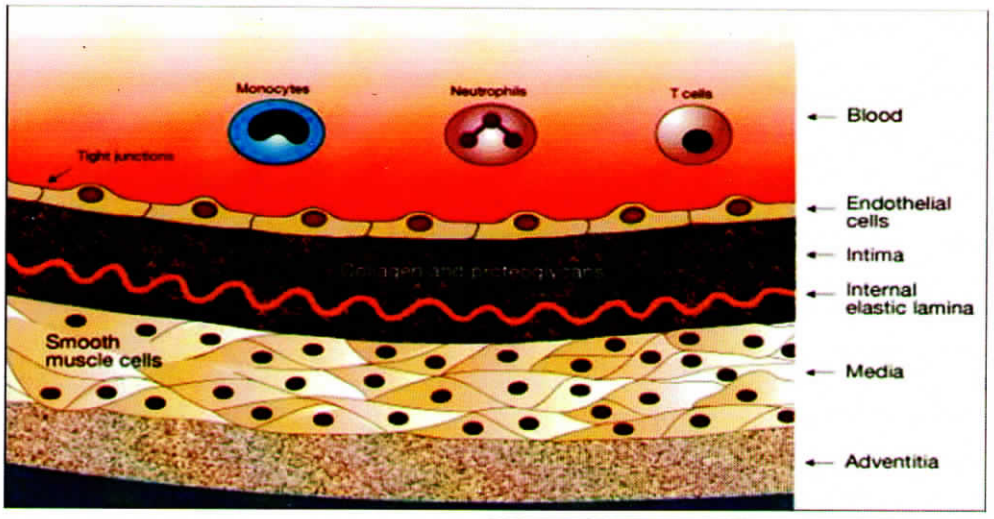

Figure2: Structure of a normal artery. (***Nature***. 2000; 407:14)

II.4. CELLS PARTICIPATING IN ATHEROSCLEROSIS

Atherosclerotic lesions mainly contain three components, namely cholesterol in the form of cholesterol esters, inflammatory and arterial wall cells and connective tissue composed of collagen, elastin and glycosaminoglycans [14]. The numerous cells involved in the pathogenesis of atherosclerosis can be broadly organized into structural elements of the arterial wall and the inflammatory cells that enter the arterial wall. The intrinsic vascular wall cells include the endothelial cells and VSMCs whereas the inflammatory cells include the monocyte/macrophages, T-lymphocytes and the mast cells [15, 16].

II.5. STAGES OF ATHEROSCLEROSIS

Atherosclerotic lesions in humans develop over a course of 50 years, beginning in the early teenage years [17]. According to the 'response to injury' hypothesis, a wide gamut of factors such as hemodynamic (hypertension), chemical (cholesterol), immunologic (immune complexes), viral, cigarette smoking etc might cause injury to the endothelium [18]. Experiments in many laboratories have failed to demonstrate that frank denudation of the endothelium occurs as a result of this injury in the early stages of atherosclerosis. Regions of denuded endothelium can be identified only in advanced atherosclerotic lesions [19]. Current knowledge suggests that the injury to the endothelium by the aforementioned factors might induce an activated state of the endothelial cells during which these cells express on the cell surface, binding sites for blood-borne monocytes. These binding sites include adhesion molecules such as the vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule (ICAM-1), P selectin etc. The endothelial cells also secrete chemokines such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) initiating the formation of the earliest lesion, the fatty streak [20, 21]. Over time and with continued irritation, these streaks progress into more complicated lesions containing an accumulation of lipids and necrotic cells [22]. These plaques are characterized by large, necrotic, highly thrombogenic lipid cores, thin fibrous caps; and increased numbers of macrophages and T lymphocytes in the shoulder regions where plaque rupture occurs [23].

Sequential steps in the pathogenesis of atherosclerosis (Figure 3)

1. The initial step in atherogenesis is the accumulation of lipoprotein particles in the arterial intima following endothelial injury.
2. The lipoproteins (depicted by the dark yellow color) gradually undergo modifications including oxidation and glycation.
3. Oxidative stress and modified LDL through local cytokine elaboration increases the expression on endothelial cells of adhesion molecules and chemokines for attachment of leukocytes (monocytes) and their migration into the arterial intima.
4. Blood monocytes, on entering the artery wall in response to chemoattractant cytokines such as monocyte chemoattractant protein 1 (MCP-1), encounter stimuli such as macrophage colony-stimulating factor (M-CSF) that can augment their expression of scavenger receptors.
5. Scavenger receptors mediate the uptake of modified lipoprotein particles and promote the development of foam cells. These modified lipoproteins are predominantly oxidized low-density lipoprotein (LDL) cholesterol [24].
6. Macrophage foam cells are a source of mediators such as more cytokines and effector molecules such as hypochlorous acid, superoxide anion (O^{2-}) and matrix metalloproteinases.
7. VSMCs from the media migrate and divide in the intima.
8. VSMCs elaborate extracellular matrix promoting extracellular matrix accumulation in the growing atherosclerotic plaque. In this manner, the fatty streak evolves into a fibro-fatty lesion.

9. Later stages of atherosclerosis involve more complicated plaques. This involves calcification and fibrosis sometimes accompanied by VSMC death (apoptosis) yielding a relatively acellular fibrous capsule surrounding a lipid-rich core that may also contain dying or dead cells and their detritus.

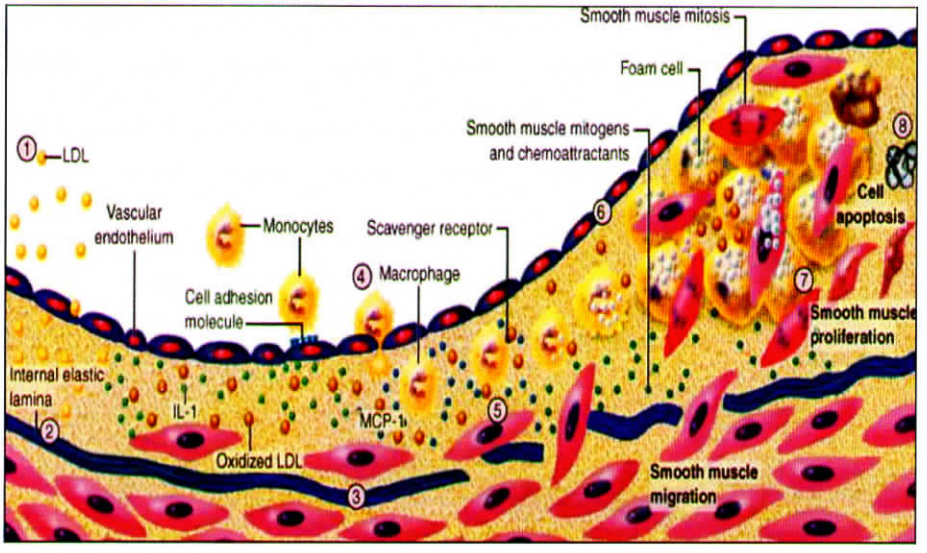

Figure 3: Schematic of the evolution of the atherosclerotic plaque. Numbers denote the sequential steps involved in the pathogenesis as mentioned in the text. (Braunwald's Heart Disease, 8th edition)

II.6. Atheroma progression and complication

The forces that maintain the stability of the fibrous cap in atheroma are of great importance and interest to scientists and clinicians alike. After formation of the fatty streak, the nascent atheroma typically evolves into a more complex lesion, which eventually leads to clinical manifestations. According to the traditional notion, fatty streaks evolve into complicated atheroma through multiplication of smooth muscle cells, which accumulate in the plaque and lay down abundant extracellular matrix. As the

lesion becomes more bulky, the arterial lumen narrows until it hampers flow and leads to clinical manifestations such as unstable *angina pectoris*, or acute myocardial infarction. Data that emerged from serial angiographic studies suggest that many coronary arterial lesions in humans develop stenoses discontinuously. Current evidence suggests that both physical disruption [25] and/ or inflammation [26] may trigger thrombosis in plaques and thus promote sudden expansion of atheromatous lesions.

II.6. A. Physical disruption in atheroma progression

Three types of physical disruption may occur.

1. Superficial erosion, or microscopic areas of desquamation of endothelial cells that form the monolayer covering the intima, results in the exposure of sub endothelial collagen and von Willebrand factor promoting platelet adhesion and activation [27]. Although common and most often asymptomatic, such superficial erosion may account for approximately one-quarter of fatal coronary thromboses.
2. Disruption of the microvessels in atherosclerotic plaques causes sudden plaque progression by thrombin generation and fibrinogen cleavage. Thrombin triggeres PDGF and TGF β release causing VSMC migration and proliferation as well as interstitial collagen synthesis [28].
3. The third mechanism of physical disruption of plaque is a fracture of the plaque's fibrous cap. Fissures in the fibrous cap will allow the coagulation factors to come in contact with tissue factor, the main pro-thrombotic stimulus found in the lesion's lipid core [29]. Although the ruptured fibrous cap causes about three-quarters of acute myocardial infarctions, most episodes probably cause no clinical symptoms. With healing, however, resorbtion of the mural thrombus and the

consequent smooth muscle accumulation and collagen accretion allow rapid evolution of a fatty lesion to one of more fibrous character.

II.6.B. Inflammation in atheroma progression

The integrity of fibrous cap is now well understood to be determined by a balance between synthesis of the matrix materials and their degradation, both of which appear strongly influenced by inflammatory stimuli. Converging lines of evidence point to the dynamic regulation of collagen levels in the plaque's fibrous cap. When inflammation prevails in the intima, smooth muscle cell production of new collagen required for repair and maintenance of the fibrous cap decreases. Meanwhile, collagen degradation increases due to overexpression of active MMPs. This results in the dissolution of the collagenous matrix of the fibrous cap rendering the structure weak, friable and susceptible to fracture when exposed to haemodynamic stresses [22, 30]. As a result of plaque rupture, the circulatory system and components of its coagulation cascade are exposed to the thrombogenic lipid core. The resulting thrombosis can precipitate a myocardial infarction or unstable angina pectoris, depending on the extent of coronary arterial occlusion [31]. Usually below the clinical threshold, evolution of the lesion most often occurs silently, leading to transition from the fatty to the fibrous atherosclerotic plaque.

II.7. RISK FACTORS FOR ATHEROSCLEROSIS

Over 300 risk factors have been associated with atherosclerosis and its major complications, coronary heart disease and cerebral stroke. Much of the progress in understanding atherosclerosis over the past 50 years has depended on the lipid hypothesis. A decade ago, lipid-lowering therapy was expected to eliminate CAD and

other forms of atherosclerosis by the end of the 20th century. LDL cholesterol undoubtedly contributes importantly to atherosclerosis in many cases, and may indeed constitute a ubiquitous permissive factor for atherogenesis. However, individuals with 'average' levels of cholesterol have proven coronary artery disease. Even extremely effective therapies targeting LDL cholesterol reduced coronary events at the most by one-third over a five-year treatment period suggesting that important pathogenic mechanisms remain unmodified by the present treatment modalities [32]. About 70 to 90 % of the risk of atherosclerotic disease can be explained by different associations between conventional risk factors, such as smoking, abnormal lipids, hypertension, diabetes, obesity, age, sex , unhealthy diet, genetic alterations and lack of physical activity [33]. Most of the above risk factors may intensify or provoke atherosclerosis through their effects on low-density lipoprotein (LDL) particles and inflammation but the molecular details of how they work are not yet known.

II.7.A. Age

The prevalence of atherosclerosis increases with age. Little is known about this factor, although it is assumed that it represents the duration of exposure to other factors.

II.7.B. Sex

Overall, men are more prone to atherosclerosis than women. It has been shown that the incidence of cardiovascular disease is twice as high in men as in women below the age of 60 years [34].

II.7.C. Elevated LDL cholesterol

There is evidence that high levels of LDL-cholesterol predispose to atherosclerosis,[35, 36] while high levels of HDL-cholesterol have a protective role [37].

II.7.D. Smoking

The association between heart disease and smoking is well established [38] and there is a dose response relationship between these two variables with a relative risk as high as 5.5 for a fatal heart attack among heavy smokers as compared to that in nonsmokers [39].

II.7.E. Hypertension

Hypertension produces a continuous trauma to the endothelium and predisposes to early stage atherosclerosis. In advanced atherosclerosis, it might contribute to plaque growth. Hypertension is associated with a two fold to three fold increase in the incidence of strokes and heart attacks as compared to normotension [40].

II.7.F. Physical inactivity

Physical activity exerts a beneficial influence on the risk factors for atherosclerosis by reducing blood pressure, weight and pulse rate, increasing the HDL cholesterol levels and lowering LDL cholesterol levels, decreasing platelet aggregability, increasing insulin sensitivity; and improving glucose tolerance [41].

II.7.G. Genetic alterations

Atherosclerosis is a disorder with multiple genetic and environmental contributions. Genetic-epidemiologic studies have identified a surprisingly long list of genetic risk factors for CAD. However, such studies indicate that family history is the most significant independent risk factor. Many Mendelian disorders associated with atherosclerosis such as **Familial hypercholesterolaemia** [42, 43], **Tangier disease** [44], **Hutchinson-Gilford syndrome** [45], **Werner's syndrome** [46] etc., have been characterized, but they explain only a small percentage of disease. Most cases of

myocardial infarction and stroke result from the interactions of multiple genetic and environmental factors, none of which can cause the disease by itself.

II.7.H. Novel risk factors

Some novel risk factors for atherosclerosis include **lipoprotein (a), homocysteine, infectious agents such as herpes virus and *Chlamydia pneumonia*** [47].

II.7.I. Diabetes mellitus

Diabetes is a powerful and independent risk factor for the development of atherosclerotic cardiovascular diseases [48]. Patients with diabetes are at a significantly high risk to develop atherosclerosis (2-6 fold) and myocardial infarction [49, 50]. Prospective studies have shown that heart attacks constitute the major cause of death in patients with diabetes [51].

II.8. TYPE 2 DIABETES MELLITUS

Diabetes mellitus is a heterogeneous group of metabolic disorders characterized by chronic hyperglycemia. There are 2 distinct forms of diabetes namely type I diabetes (insulin-dependent diabetes mellitus) and type 2 diabetes (non insulin dependent diabetes). Type 1 diabetes is an autoimmune disorder that results in β-cell death in the pancreas and a dramatic decrease in insulin secretion [52, 53]. Type 2 diabetes has a slower onset and is characterized by insulin resistance in adipose tissue, liver, and skeletal muscle, as well as impaired β-cell function [54, 55].

Five to ten percent have type 1 and 90% to 95% have type 2 diabetes mellitus.The incidence of type 2 diabetes is likely to rise as a consequence of lifestyle patterns contributing to obesity [56]. WHO has classified type 2 diabetes as pandemic with 194

million diabetic patients for the year 2003 which is projected to reach 333 million by the year 2025 [57]. Cardiovascular physicians are encountering many of these patients because vascular diseases are the principal causes of death and disability in people with diabetes. The macrovascular manifestations include atherosclerosis and medial calcification whereas microvascular consequences include retinopathy and nephropathy, the major causes of blindness and end-stage renal failure respectively. Factors contributing to the pandemic of type 2 diabetes include an aging population, increased abdominal obesity, unhealthy diet, decreasing level of physical activity, genetic associations etc [58].

II.8.A. PATHOPHYSIOLOGY OF DIABETIC VASCULAR DISEASE

Despite their differing etiologies, both forms of diabetes increase the risk of developing atherosclerosis by 2–4 fold [59]. Plausible biologic mechanisms for the increased risk for atherosclerosis in type 2 diabetes include endothelial dysfunction, platelet hyperactivity, VSMC dysregulation, propensity for adverse arterial remodeling, enhanced cellular and matrix proliferation after arterial injury and impaired fibrinolysis with a tendency for thrombosis and inflammation. The metabolic abnormalities that characterize diabetes namely hyperglycemia, insulin resistance and increased free fatty acids all contribute to dysfunction of vascular cells especially the endothelial cells *via* various mechanisms [6]. In patients with diabetes and those with metabolic syndrome, atherosclerosis is the cause of a majority of cardiovascular events and interestingly, the rate of atherosclerosis is also accelerated. The presence of diabetes (both type 1 and type 2) increases the risk of CVD beyond that seen with metabolic syndrome alone and one notable fact is that, the

presence of the metabolic syndrome is less common in type 1 diabetes [60, 61]. Therefore, the unique thing that distinguishes diabetes from metabolic syndrome and might underlie the increased risk of atherosclerotic CVD in both types 1 and 2 diabetes may be hyperglycemia, the metabolic abnormality associated with the diabetic state and which is common to both type 1 and type 2 diabetes.

II.8.B. HYPERGLYCEMIA AND ATHEROSCLEROSIS

A strong correlation exists between hyperglycemia and both micro- and macrovascular diseases [59, 62-64]. Hyperglycemia is now recognized as an important regulator for the accelerated rate of development of atherosclerotic vascular lesions [2, 3]. Prolonged exposure to hyperglycemia is now recognized as the primary casual factor in the pathogenesis of diabetic complications [65, 66]. Strong epidemiological evidence supports an association between glycemic control and CVD risk [49]. The United Kingdom Prospective Diabetes Study (UKPDS) provided additional insights into the relationship between glycemic control and CVD in patients with type 2 diabetes, indicating a linear relationship between HbA1c and CVD endpoints, particularly myocardial infarction [67].

II.8.C. MECHANISM BY WHICH HYPERGLYCEMIA PROMOTES ATHEROSCLEROSIS

Hyperglycemia induces a large number of alterations in vascular tissue especially the vascular endothelium that potentially promote accelerated atherosclerosis. In general, the adverse effects of hyperglycemia can cause vascular dysfunction such as endothelial

dysfunction either by generating toxic and reactive metabolites or by altering intracellula signaling pathways **(Figure 4).**

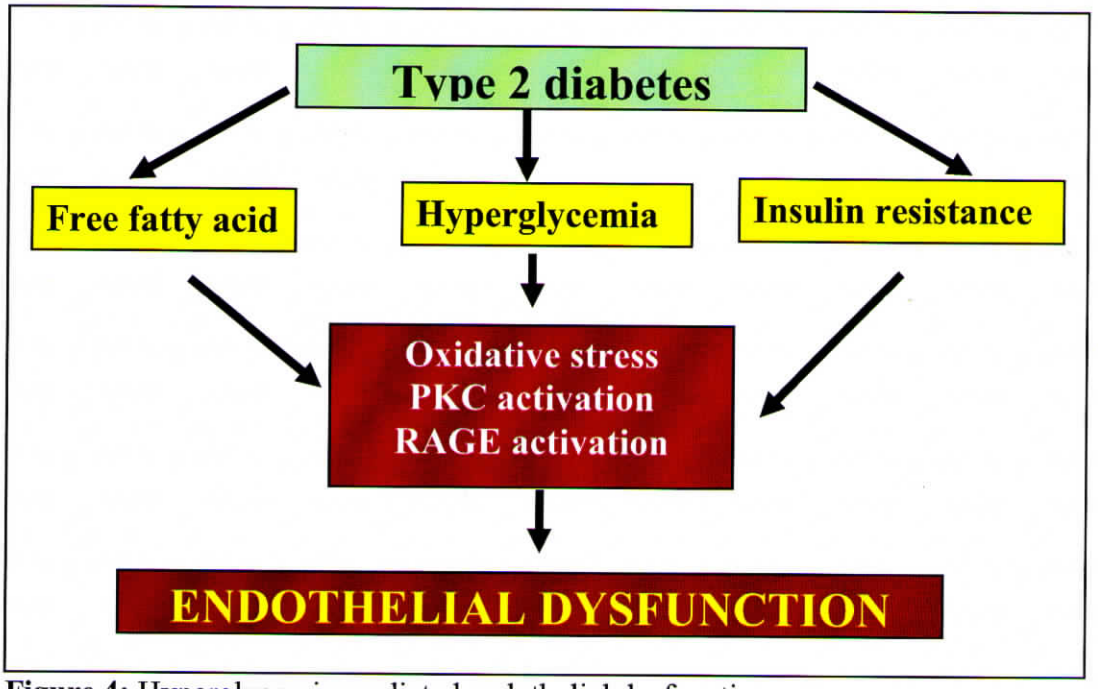

Figure 4: Hyperglycemia mediated endothelial dysfunction.

Three well researched mechanisms on how hyperglycemia produces most of the pathological alterations including vascular derangements and dysfunction that is observed in the macrovasculature of diabetic animals and humans is as follows: 1) Advanced glycation end product formation 2) oxidative stress and 3) protein kinase C (PKC) activation.

II.8.C.1. Advanced glycation end product formation

One of the important mechanisms responsible for accelerated atherosclerosis in diabetes is the non-enzymatic reaction between glucose and proteins or lipoproteins in arterial walls, collectively known as Maillard, or browning reaction [68]. Glucose forms chemically reversible early glycosylation products with reactive amino groups of circulating or vessel wall proteins (Schiff bases), which subsequently rearrange to form the more stable Amadori-type early glycosylation products. Some of the early glycosylation products on long-lived proteins such as vessel wall collagen continue to undergo complex series of chemical rearrangement to form advanced glycosylation end products (AGEs) which are stable and virtually irreversible. AGEs accumulate continuously on long-lived vessel wall proteins with aging and at an accelerated rate in diabetes which is subject to the glucose concentration and duration of exposure [69]. AGEs formation also increases substantially during situations in which the local redox potential has been shifted to favor oxidant stress [70-72]. AGEs can accelerate the atherosclerotic process by diverse mechanism such as the non-receptor dependent and receptor-mediated pathways.

A. Non-receptor mediated mechanisms

Non-enzymatic glycosylation of proteins and lipoproteins can interfere with their normal function by disrupting molecular conformation, altering enzymatic activity, and interfering with receptor recognition. Thus, changes in the normal physiology of proteins that are relevant to atherogenesis, may promote atherosclerosis in diabetic individuals. An ideal example is interference of the normal physiology of LDL particle. LDL

glycosylation was found to be increased in correlation with glucose levels, and AGE-ApoB levels were 4-fold higher in diabetic patients [73, 74]. Glycosylation of ApoB significantly impairs receptor-mediated uptake of glycosylated LDL and *in vivo* clearance compared to native LDL [75]. Consequently there is an increased uptake of glycosylated LDL by non-specific (scavenger) receptor present on monocyte- derived macrophages stimulating foam cells formation [76]. The recognition of glycated LDL by the scavenger receptor pathway is thought to promote intracellular accumulation of cholesteryl esters and thereby atherosclerosis. Glycation also confers increased susceptibility of LDL to oxidative modification, a critical step in atherogenesis [77].

B. Receptor-mediated mechanisms

The cellular interactions of AGEs are mediated through a specific receptor for AGE products on cell surfaces called the receptor for AGE (RAGE). RAGE is a member of the immunoglobulin superfamily of receptors and is expressed in all cells relevant to the atherosclerotic process including monocyte-derived macrophages, endothelial cells, and smooth muscle cells [78, 79]. AGE interaction with RAGE on endothelial cells cause induction of oxidative stress and consequent VCAM-1 expression [80] and NF-κB activation [81]. RAGE-AGEs interaction can result in reduced endothelial barrier function [82], with increased permeability of endothelial cell monolayers [83]. Thus, the interaction of AGEs with RAGE-bearing endothelial cells can mediate initiating events in atherogenesis. It has also been shown that binding of soluble AGEs to RAGE-bearing monocytes induces chemotaxis [84] followed by mononuclear infiltration through an intact endothelial monolayer [85]. Pathological studies of human atherosclerotic plaques showed infiltration of RAGE-expressing cells in the expanded intima [86]. Monocyte-

macrophage interaction with AGEs result in the production of mediators such as IL-1, TNF-α, PDGF, and IGF-1 [87, 88], all of which are reported to play an important role in atherosclerosis [24]. In diabetic model of atherosclerosis-prone apolipoprotein E (apoE) knock out mice, blockade of AGE-RAGE interaction using a truncated soluble extracellular domain of RAGE resulted in a striking suppression of lesions in diabetic mice and the effects were independent of glucose and lipid levels [89].

II.8.C.2. Oxidative stress

Among the sequelae of hyperglycemia, oxidative stress has been suggested as a potential mechanism for accelerated atherosclerosis. The major mechanism by which hyperglycemia increases oxidative stress appears to be *via* reactive oxygen species (ROS) produced by the proton electrochemical gradient generated by the mitochondrial electron transport chain [70]. The other mechanisms involves the transition metal catalyzed autoxidation of free glucose and protein-bound Amadori products yielding superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2) which damage cellular proteins [90] and mitochondrial DNA [91]. The increased oxidant stress can promote leukocyte adhesion to the endothelium while inhibiting its barrier function [92]. ROS modifies endothelial function by a variety of mechanisms. These include direct effects on the endothelium such as peroxidation of membrane lipids, activation of transcription factors (e.g. NFκB and AP-1) leading to upregulation of adhesion molecules and chemokines to leukocytes and interference with the availability of NO. The indirect effects include increasing the oxidation of LDL, formation of AGEs and the activation of platelets and monocytes [6]. There is also evidence that hyperglycemia may compromise natural antioxidant defenses.

Hyperglycemia impairs endothelial free radical scavenging by reducing the activity of the pentose phosphate pathway and thus decreasing the availability of NADPH to the glutathione redox cycle [93].

II.8.C.3. Protein kinase C (PKC) activation.

PKC, a family of at least 12 isoforms of serine and threonine kinases, are critical intracellullar signaling molecules that can regulate many vascular functions such as permeability, vasodilator release, endothelial activation and growth factor signaling [94, 95]. Hyperglycemia mediated activation of the protein kinase C (PKC) system has been implicated in the pathogenesis of diabetic complications [96]. High glucose concentrations activate PKC by increasing the formation of diacylglycerol (DAG), the major endogenous cellular co-factor for PKC activation [97]. Activated PKC in the vasculature can cause vascular damage that includes increased permeability [98], NO dysregulation [99], increased leukocyte adhesion[100] and alterations in blood flow [101]. PKC activation can also impact other signaling pathways such as MAP kinase and transcription factors [102]. PKC activation is also found to increase the expression of TGF-β, an important growth factor that can regulate extracellular matrix production and decrease MMP synthesis [103].

II.8.C.4. Cross talk among the above mechanisms

The above mechanisms of vascular damage are tightly linked to each other and are not independent. For example, hyperglycemia-induced oxidative stress promotes the formation of AGE products and PKC activation. The clear example of interactions among the different pathways is the formation of oxidants. Elevated levels of oxidants can be derived from increased mitochondrial superoxide production in the diabetic state [70].

Some AGEs are oxidants and contribute to glycoxidation and lipid peroxidation [104, 105]. AGE binding to its cellular receptors can also increase oxidant production by stimulating NADP(H) oxidase [81]. Activation of PKC can affect the production of oxidants and AGE by activation of oxidases such as NADP(H) oxidase [106]. These mechanisms can also interact and enhance one others' biological effects through various signaling pathways activated by glucotoxins. For example, extracellular AGEs can bind to cellular receptors such a RAGE, which activates signaling enzymes such as PKC and MAP kinase, thereby altering vascular cell function and survival [107]. Oxidative stress can also activate PKC by increasing DAG formation [108]. Other signaling molecules such as p38 or Jun-N-terminal MAP kinase are known to be activated by oxidants causing enzymatic and transcriptional changes as well as alteration in cellular survival [109, 110].

Therefore, hyperglycemia can cause a plethora of biochemical and biological changes in the macrovasculature including endothelial cells promoting atherogenesis. Given that vascular endothelium plays a pivotal role in maintaining vascular homeostasis and in the initiation of atherosclerotic process (as per the 'response to injury' hypothesis), hyperglycemia mediated injury or activation of the vascular endothelium may become detrimental to the healthy vasculature.

II.8.D. ENDOTHELIAL DYSFUNCTION

The vascular endothelium comprises a diverse population of cells that specialize in response to genetic programmes and environmental cues to take on distinct roles in different vessels, tissues and organs and in response to pathophysiological stresses.

It is a continuous cellular monolayer lining the entire circulatory system with an enormous range of homeostatic roles. The functions of endothelium are mediated by secreting a wide range of biologically active substances broadly categorized as vasodilators and vasoconstrictors, pro and anti-thrombotic factors, fibrinolytic activators and inhibitors, leukocyte adhesion molecules, cytokines and chemokines **(Table 1)** [111].

Vasodilators	**Vasoconstrictors**
NO,PGI2, EDHF, C-natriuretic peptide	ET-1, Ang II, Endoperoxide(PGH2), TXA2
Antithrombotic	**Prothrombotic**
t-PA, PGI2, NO	PAI-1, TXA2
Growth inhibitors	**Growth promoters**
NO, PGI2, C-natriuretic peptide	Superoxide radicals, ET-1, Ang II
Inflammation inhibitors	**Inflammation promotors**
NO	Super oxide and other radicals, TNF-α

Table 1: Substances synthesized by endothelium and their biological functions

The endothelial cells actively regulate vascular tone and permeability [112], maintains the balance between coagulation and fibrinolysis; regulates the composition of the subendothelial matrix; extravasation of leukocytes [113] and the proliferation of VSMCs. Normally, the endothelium actively decreases vascular tone, a process in which NO plays a key role [114]. In addition, the endothelial cells normally inhibit platelet adhesion and aggregation by producing prostacyclin and NO, limit activation of coagulation cascade by the thrombomodulin-protein C and heparin sulphate- antithrombin III interactions, and regulate fibrinolysis by producing t-PA and its inhibitor, PAI-1[115]. Therefore in

healthy conditions, there is a balance between the various biologically active mediator synthesized by the endothelium **(Figure 5).**

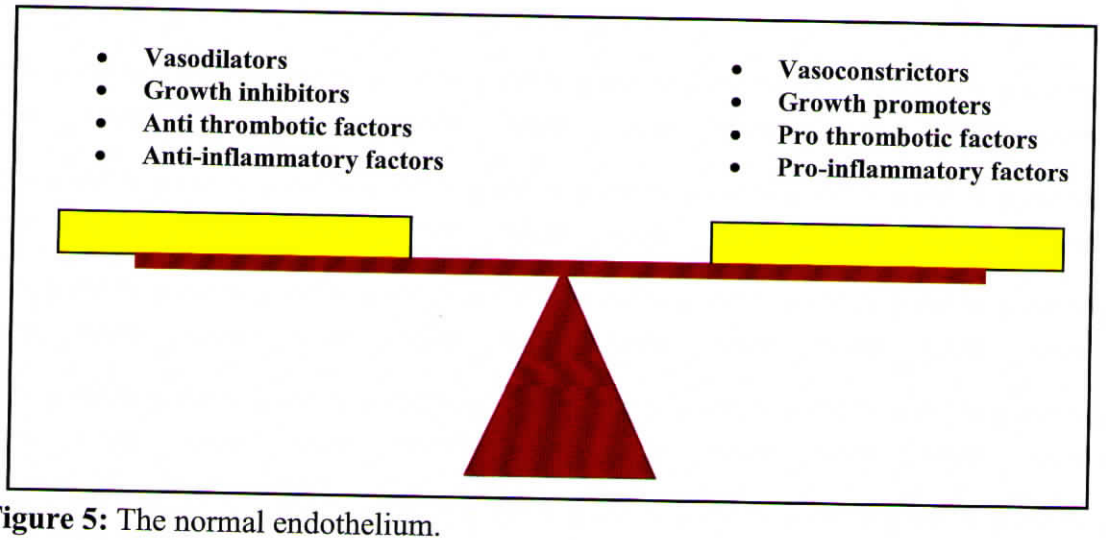

Figure 5: The normal endothelium.

II.8.E. Endothelial dysfunction and its consequences

Hyperglycemia converts endothelial cell from an inhibitor of atherosclerosis to a participant in the process. Endothelial dysfunction is thought to be a major early step in the development of accelerated atherosclerotic lesions and can be seen even before the development of clinically manifested vascular disease. The vascular endothelium is considered dysfunctional or activated by hyperglycemia when its properties, either basal state or after stimulation, have changed in a way that is inappropriate with regard to the preservation of organ function. It can be a consequence of imbalance between relaxing and contracting factors, pro and anti-coagulants or growth promoting and inhibiting agents **(Figure 6).**

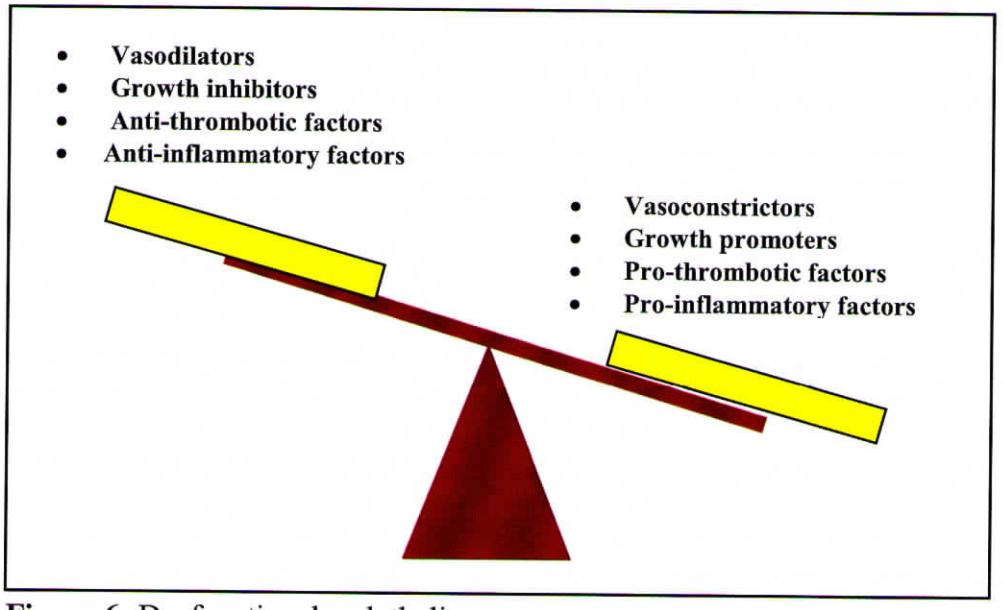

Figure 6: Dysfunctional endothelium.

NO protects the blood vessel from endogenous injury—ie, atherosclerosis—by mediating molecular signals that prevent platelet and leukocyte interaction with the vascular wall and inhibit vascular smooth muscle cell proliferation and migration [116-118]. Conversely, the loss of endothelium- derived NO permits increased activity of the proinflammatory transcription factors such as NFκB, AP-1 etc. leading to an upregulation of chemokines (MCP-1, IL-8 etc.), cytokines (IL-1, IL-6, IL-10 etc.) and cell adhesion molecules (Selectins, ICAM-1, VCAM-1 etc) promoting inflammation [119]. These actions cause monocyte and VSMC migration into the intima and formation of macrophage foam cells, characterizing the initial lesions of atherosclerosis [22, 120, 121]. Endothelial dysfunction, as represented by impaired endothelium-dependent NO-mediated relaxation, occurs in cellular and experimental models of diabetes [122-125]. Similarly, many, but not all, clinical studies have found that endothelium-dependent

vasodilation is abnormal in patients with type 1 or type 2 diabetes [126-129]. Thus, decreased levels of NO in diabetes may underlie its atherogenic predisposition. The bioavailability of NO reflects a balance between its production *via* NOS and its degradation particularly by oxygen-derived free radicals [130, 131]. Many of the metabolic derangements known to occur in diabetes, including hyperglycemia, mediate abnormalities in endothelial cell function by affecting the synthesis or degradation of NO [132]. Endothelial dysfunction is also accompanied by decreased local availability of NO, increased oxidation of LDL cholesterol, altered intracellular signalling pathway, hyper-adhesiveness of blood leukocytes, increased permeability to monocytes and lipoproteins. Endothelial dysfunction results in increased platelet adherence and increased smooth muscle cell migration and proliferation [5, 6]. These functional changes in the vascular endothelium can initiate the process of atherogenesis and progression of atheroma. Endothelial dysfunction also results in increased tissue factor expression that can initiate blood coagulation *via* the extrinsic pathway. At the same time it can cause inhibition of fibrin degradation by reducing the t-PA levels while increasing PAI-1 levels promoting coagulation and thrombosis. Thus there is a risk of thrombosis complicating plaque rupture **(Figure 7)** [56].

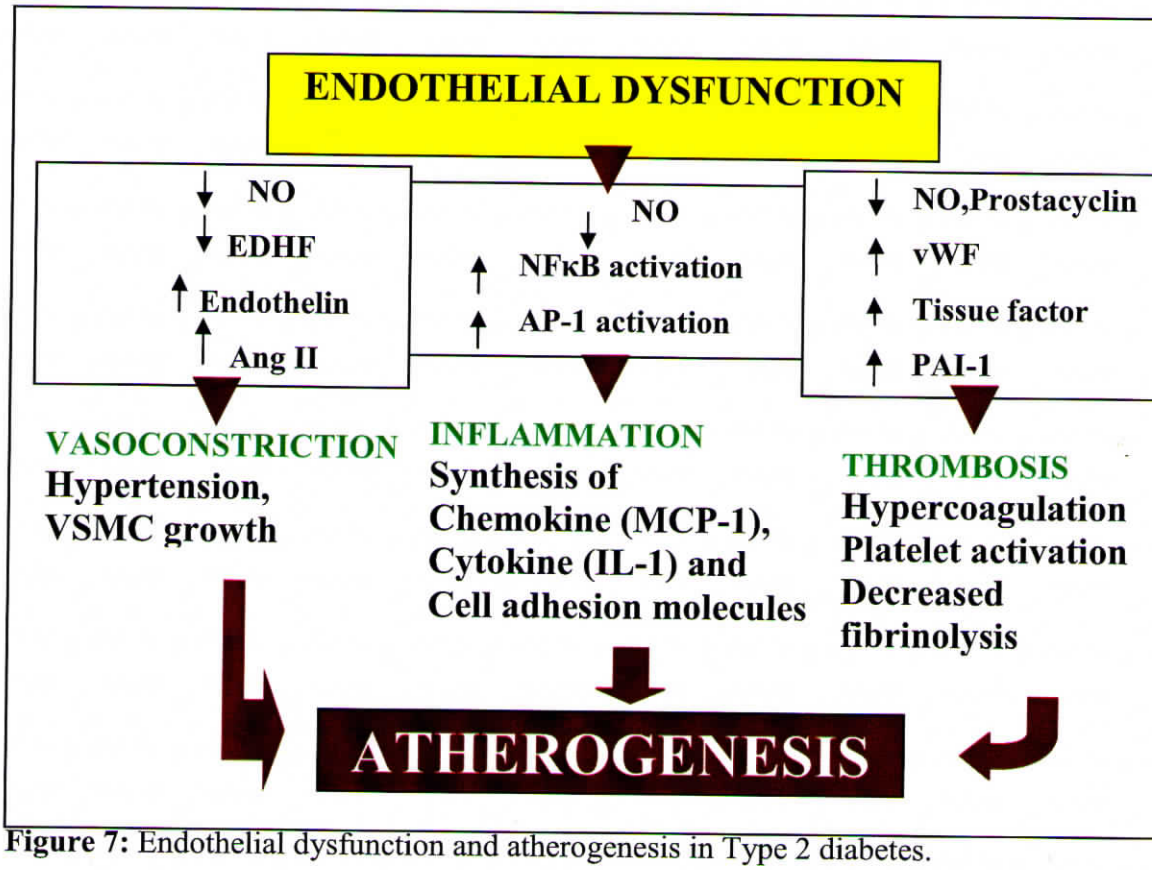

Figure 7: Endothelial dysfunction and atherogenesis in Type 2 diabetes.

Thus, hyperglycemia mediated endothelial dysfunction with the loss of vascular protective effect is a major complication in type 2 diabetes that may increase the risk of atherosclerosis in diabetes.

II.8.F. MONOCYTE CHEMOATTRACTANT PROTEIN-1 (MCP-1)

A preponderance of evidence from clinical and experimental studies supports the notion that inflammation plays an important role in a wide range of cardiovascular diseases and has focused attention on the signals that initiate cellular infiltration of vascular tissues.

Chemokines (chemotactic cytokines) are small heparin binding proteins that direct the migration of circulating leukocytes to sites of inflammation or injury [133, 134]. There are approximately 50 human chemokines which are divided into three main families according to their structure and function. The largest family is the CC chemokines, in which the first two of the four amino terminal cysteine residues are adjacent to each other. Genes for CC chemokines (β chemokines) are found on chromosome 17. One interesting fact is that, hyperglycemia mediated endothelial dysfunction can result in the upregulation of MCP-1 , a CC chemokine involved in the transmigration of blood monocyte form lumen into the arterial intima, an early critical event in atherogenesis. MCP-1 or CCL2, a monomeric polypeptide of 9000 to 15,000 Da molecular weight exhibits its most potent activity toward monocytes and are found at sites of chronic inflammation [135]. It is one of the most thoroughly characterized chemokines which is a potent agonist for monocytes, memory T cells, and basophils. Other members of the CC family include RANTES (CCL5), macrophage inflammatory protein 1 α (MIP-1 α) (CCL3), and MIP-1β (CCL4) [136]. The function of MCP-1 is totally dependent on its receptors. In monocytes, three different receptors namely CCR1,CCR2, and CCR5, have been identified, the former two of which were the only ones present in granulocytes [137, 138]. It has been proposed that CCR2 serves as the principal MCP-1 receptor. The primary function of MCP-1 in atherogenesis is to help the rolling monocytes to adhere firmly onto the endothelial cells expressing E-selectin [32] and in the transmigration of monocyte into the subendothelial space [139]. Besides monocyte recruitment, MCP-1 activates monocytes and induces the expression of tissue factor (TF), superoxide anions, and proinflammatory genes [140]. MCP-1 is produced by various cell types such as

endothelial cells, VSMCs and macrophages in response to various stimuli such as cytokines[141], minimally modified LDL [142], Ang II[143], homocysteine [144], shear stress [145], high glucose concentrations [146, 147] and activated platelets[148]. MCP-1 has been found to be a key molecule in reperfusion injury [149] and in restenosis in patients after PTCA [150-152]. MCP-1 is also related to the pathogenesis of acute coronary syndrome through its ability to enhance coagulability [153]. MCP-1 mediated inflammatory disorders are also involved in other treatment-intractable cardiovascular diseases, such as posttransplantation arteriosclerosis, vascular remodeling owing to hypertension, myocarditis/cardiomyopathy and cardiac dysfunction and remodeling after myocardial infarction [154-159].

II.8.G. MCP-1 IN ATHEROGENESIS

The hallmark of early atherosclerotic lesions, the 'fatty streaks' is composed of lipid-laden macrophages called foam cells. Several lines of evidence now support the hypothesis that MCP-1 plays a critical role in recruiting these monocytes into early atherosclerotic lesions. MCP-1 is found to be present in macrophage-rich atherosclerotic plaques in humans [160]. Oxidized lipids have long been implicated as mediators of atherosclerosis and foam cell formation [161]. Studies by Cushing *et al.* have demonstrated that minimally oxidized-LDL induces MCP-1 production in vascular wall cells such as endothelial cells and smooth muscle cells [142]. Studies in transgenic mice overexpressing MCP-1 and in knock out mice for MCP-1 or its receptor, CCR2, provided strong evidence that MCP-1 is involved in the recruitment of monocytes into the arterial intima. The overexpression of MCP-1 in specific tissues caused a localized infiltration of

monocyte/macrophages [162]. In bone marrow transplantation studies, overexpression of MCP-1 in vessel wall macrophages led to increased foam cell formation and increased atherosclerosis [163]. Deletion of MCP-1 in LDL receptor-null mice attenuated the progression of diet-induced atherosclerosis **(Figure 8)** [164].

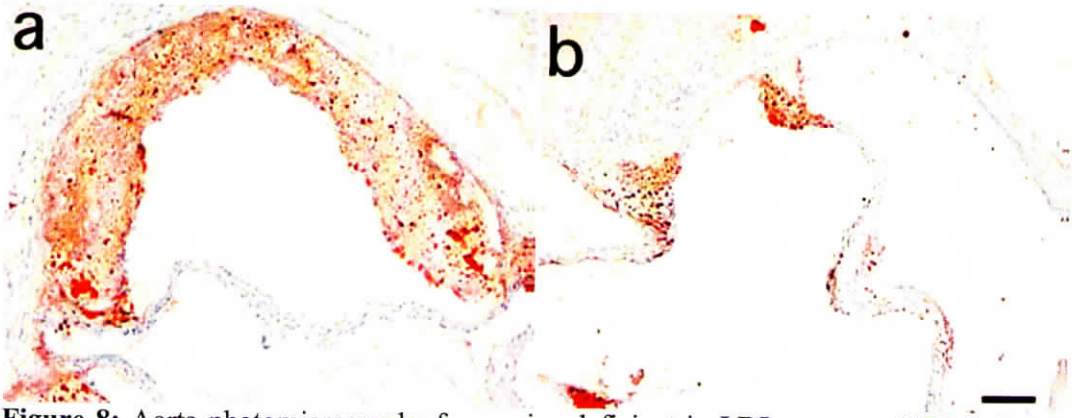

Figure 8: Aorta photomicrographs from mice deficient in LDL receptor (LDLr) (Panel A) and in double knockout (LDLr and MCP-1, Panel B) animals, showing a significant reduction in the area of atheromatous plaque in those animals deficient in MCP-1. (***Mol Cell.*** 1998;2: 275–281. Reproduced with permission)

Similar results were reported in MCP-1–null mice expressing human apolipoprotein B [165]. The deletion of MCP-1 receptor CCR2 in apoE deficient mice gave significant protection from both macrophage accumulation and atherosclerotic lesion formation when put on high-fat diet [166]. Similar studies in mice fed a regular chow diet revealed that CCR2-/- mice were more resistant to the development of atherosclerosis than wild-type mice [167]. In contrast, mice deficient in CCR5, which is activated by MIP-1α and RANTES but not by MCP-1, were not protected against atherosclerosis [168]. These studies provide strong evidence that activation of CCR2, presumably by MCP-1, contributes to foam cell formation, one of the earliest manifestations of atherosclerosis. Although most work has focused on MCP-1, other chemokines may also play a role. For example, Met-RANTES, a chemokine receptor antagonist that blocks CCR1 and

CCR5, significantly reduced lesion progression in atherosclerotic mice [169]. The importance of inflammation in the later stages of atherosclerosis, especially plaque rupture, has been emphasized in several recent reviews [32]. The extent to which chemokines such as MCP-1 contribute to the retention and activation of macrophages in advanced lesions is unclear.

In animal and human atherosclerotic lesions, 80% of leukocytes are monocytes/macrophages and 10 to 20 % of them are memory T-lymphocytes [170]. Atheroma-forming cells (endothelial cells, smooth muscle cells, and macrophages) express MCP-1 and CCR2, and this pathway is active in atherosclerotic lesions [171]. Abrogation of the MCP-1/CCR2 pathway inhibits the early development of atherosclerotic lesions in mice [164, 166]. Anti–MCP-1 therapy not only attenuated atherosclerosis but also limited the progression and destabilization of established atherosclerosis suggesting that blockade of the MCP-1/CCR2 pathway might lead to reductions in atherosclerotic complications [172, 173]. Oxidative stress, oxidized inflammatory lipids, and redox-sensitive transcription factors (NF-kB, AP-1, etc) reportedly contribute to increased expression of MCP-1. Further, activation of the MCP-1/CCR2 pathway induces adhesion molecules [174], proinflammatory cytokines, chemokines, and matrix metalloproteinases [175, 176] and thus accelerates atherosclerosis in hypercholesterolemic animals [163, 177]. More importantly, MCP-1 can induce expression of tissue factor and inflammatory cytokines such as IL-6 in human arterial smooth muscle cells [140]. These findings suggest that MCP-1 contributes not only to vascular inflammation but also to the development of atherosclerosis, plaque destabilization and thrombosis which can result in acute coronary syndrome **(Figure 9).**

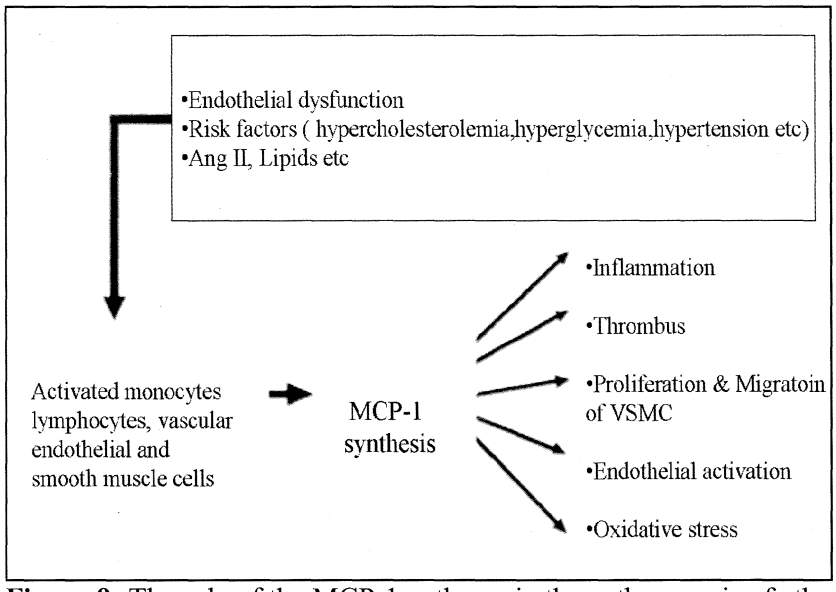

Figure 9: The role of the MCP-1 pathway in the pathogenesis of atherosclerosis, plaque destabilization and thrombosis. (***Hypertension.*** 2003; 41(3): 834. Reproduced with permission)

II.8.H. MCP-1 IN DIABETES

Numerous studies have documented glucose-induced endothelial dysfunction that may promote atherogenesis, such as increased production of cell adhesion molecules and cytokines (IL-1β and IL-8), enhanced leukocyte adhesion and decreased cell proliferation and apoptosis [178-185]. Recently it was reported that in human aortic endothelial cells, HG increases MCP-1 mRNA and protein synthesis [146, 186]. Studies have also demonstrated that in vascular endothelial cells, HG induces an increase in MCP-1 production *via* p38 MAPK pathway [147]. In patients with diabetes mellitus, the circulating levels of MCP-1 is increased and this situation can possibly augment the recruitment of monocytes to the vessel wall [187]. Serum MCP-1 levels correlates significantly with fasting plasma glucose, HbA1c, triglyceride, body mass index, and high sensitivity CRP [187]. It has been observed that the expression levels of CCR2, CD36, and CD68 on monocytes were upregulated by MCP-1 stimulation [187]. Analysis

of gene expression of MCP-1 and RAGE in endothelial biopsy obtained from patients with type 2 diabetes has revealed increased levels of MCP-1 and RAGE transcripts. These data additionally confirm the existence of an inflammatory process attributable to hyperglycemia [188]. Taken together, the above results strengthen the notion that hyperglycemia accelerates atherosclerosis by causing endothelial dysfunction and consequent proinflammatory state.

Hence it can be reasonably suggested that *in vivo* hyperglycemia stimulates the release of MCP-1 in both vascular wall cells (endothelium and VSMC) and monocytes thereby contributing to the amplified recruitment, accumulation and activation of monocyte/macrophages in the atherosclerotic lesions.

II.8.I. MCP-1 GENE REGULATION

The molecular mechanism leading to MCP-1 gene expression in endothelial cells during hyperglycemic conditions in type 2 diabetes mellitus is yet to be elucidated. The transcriptional and translational regulation of MCP-1 gene and the precise signaling mechanisms by which HG stimulates MCP-1 gene expression in aortic endothelial cells are still not well understood.

HG concentration *via* several mechanisms such as oxidative stress, PKC activation and AGE formation cause endothelial cell dysfunction resulting in increased MCP-1 expresion. This MCP-1 gene induction is mediated by various signalling events that

involve ROS and p38 MAPK which can activate the transcriptional regulators of MCP-1 gene such as NFκB and AP-1 in human endothelial cells [146, 147, 186, 189, 190]. PKC enzymes are found to be important mediators of biochemical and functional changes in diabetic vasculature of both rats and mice [5]. Interestingly enough, MCP-1 itself has been shown to induce activation of MAP-kinases such as ERK, JNK and p38 MAPK in HUVEC [191], HG stimulated MCP-1 synthesis in human endothelial cells can also cause an increased monocyte adhesion and transmigration [146, 186]. These findings demonstrate that HG increases MCP-1 expression in endothelial cells contributing to the characteristic monocyte adhesion and infiltration seen in atherogenesis. It is generally accepted that hyperglycemia can induce multiple changes in the cellular metabolism and signaling [192]. There are compelling data that chronic inflammation and activation of NFκB is a significant feature of secondary diabetic complications. Several cell types such as vascular endothelial cells, VSMC, lens epithelial cells etc. when cultured in high glucose concentration show increased NFκB activity [186, 193, 194]. Recently, activated NFκB was found to be associated with atherosclerotic plaques in patients with type 2 diabetes [195]. The signalling pathway responsible for MCP-1 gene induction in primary endothelial cells when stimulated with $NiCl_2$ was found to be IKKβ/IκBα/ NFκB pathway [196]. The transcription factor, AP-1 is also suggested to be involved in HG induced MCP-1 expression in human endothelial cells [190]. Both transcription factors, NFκB and AP-1 were found to be activated by ROS that is genenrated in endothelial cells when exposed to HG [190]. Other than HG, a variety of stimuli such as TNFα [197] IL-1 [198], IL-4 [199], LPS [200] etc. ,have been shown to induce MCP-1 gene expression in endothelial cells . It has been reported that the activation and cooperative binding of both

NFκB nd AP-1 transcription factors are required for maximal cytokine induction of MCP-1 gene in HUVECs [198]. Two NFκB, one AP-1 and one Sp-1 binding sites have already been characterized upstream of MCP-1 gene promoter in humans, mice and rats [201, 202]. Another study has found that the transcription factor STAT1 (signal transducers and activators of transcription factor 1) was involved in IL-4 induced MCP-1 expression in HUVECs [199].

Given the role of MCP-1 in the initiation and progression of atherosclerosis and the fact that it is upregulated in type 2 diabetes, it is reasonable to investigate the potential value of MCP-1 not only as a therapeutic target but also as a biomarker (both diagnostic and prognostic) for the increased risk for atherosclerosis in type 2 diabetes.

II.8.G. MOLOECULES EXPLORED FOR THE MODULATING EFFECT ON HG INDUCED MCP-1 EXPRESSION

The molecules for the present study were selected based on their anti-inflammatory and anti-oxidant properties. Molecules were also selected based on their known inhibitory effect on transcription factors such as NFκB and AP-1.

II.7.G. 1. LOSARTAN

Losartan, with an IUPAC name of 2-*n*-butyl-4-chloro- 5-hydroxymethyl-1-((2'-(1*H*-tetrazol-5yl)(biphenyl-4yl)methyl)- imidazole potassium salt is a strong non-peptide anti-hypertensive agent and exerts its action by specific blockade of angiotensin II (Ang II) receptors [203]. Ang II is known to increase MCP-1 synthesis in endothelial cells and this effect can be attenuated by losartan [204]. Xie *et al.* found that losartan reduced MCP-1

expression in aortic tissues of 2K1C hypertensive rats suggesting a molecular link between hypertension and the development of atherosclerosis [205]. Losartan was also shown to have anti-atherosclerotic effect in patients with hypertension [206]. But the benfical effect of losartan in abating the risk for atherosclerosis in diabetes is not known. Flammer *et al.* found that losartan improved diabetes induced endothelial dysfunction, an early manifestation of atherosclerosis. The study also demonstrated that this effect was independent of losartan's blood pressure-lowering effect and was probably caused by an anti-oxidative effect [207]. No studies have been done so far to demonstrate whether losartan has any effect on its own independent of Ang receptor blockade, in modulating HG induced MCP-1 expression in any type of cells. This assumes significance because in hypertensive patients with diabetes there are numerous other stimuli such as hyperglycemia which mediates its effect independent of AngII and can strongly induce MCP-1 expression in endothelial cells and monocytes, the cell types that participate in atherosclerosis. Therefore the effects of losartan itself in modulating HG induced MCP-1 synthesis in aortic endothelial cells independent of its Ang II receptor blockade was explored.

II.8.G. 2. Flavonoids

Flavonoids are polyphenolic compounds naturally present in fruits and vegetables in glycosylated forms. There are six classes of flavonoids: flavonols, flavones, isoflavones, flavanones, flavan-3-ols and anthocyanins. There is increasing evidence that flavonoids may play a protective role in reducing CVD risk [208]. Studies have shown that flavonoids have anti-inflammatory activity, decreasing the expression of cell adhesion molecules, VCAM-1, ICAM-1, E-selectin and PCAM-1 [209-211]. The

compounds used in the present study namely genistein and quercetin belongs to the isoflavone class and the flavonol class respectively.

II.8.G. 2A. GENISTEIN

Genistein is one of the most abundant isoflavones and soybeans are their exclusive dietary source. A number of cardioprotective benefits have been attributed to dietary isoflavones including a reduction in the levels of LDL cholesterol as well as susceptibility of the LDL particle to oxidation, inhibition of pro-inflammatory cytokines, cell adhesion proteins and inducible nitric oxide production and the inhibition of platelet aggregation and an improvement in vascular reactivity [212]. Isoflavone aglycone-rich extract without soy protein has been found to attenuate atherosclerosis development in cholesterol-fed rabbits primarily by inhibiting the oxidation of LDL, thereby exerting an anti-atherosclerotic effect [213] whereas genistein as such does not inhibit the progression of established atherosclerotic lesions in older apoE deficient mice [214]. Genistein is considered to have many health benefits which are mediated by its agonist activity for the β-subtype of eostrogen receptor [215]. Effects of genistein were also reported as dependent on its inhibitory activity of tyrosine kinase enzymes involved in the control of many cellular processes [216]. However, effects independent of this activity have been also demonstrated [217]. Genistein is found to completely block leptin-induced ROS generation, CPT-1 activation and MCP-1 synthesis in BAECs [218]. Genistein was also found to inhibit secretion of MCP-1, ICAM-1 and VCAM-1 in activated human endothelial cells by modulating tyrosine kinase activity [219, 220]. Recently, genistein has generated considerable interest because of its modulating role in

carbohydrate and lipid metabolism [221]. Despite the above known biological effects of genistein, no studies have been done to explore the anti-oxidant and anti-inflammatory action of genistein in endothelial cells exposed to different experimental settings that simulate diabetes conditions such as hyperglycemia. Therefore, the effect of genistein in modulating HG induced MCP-1 synthesis in aortic endothelial cells was explored.

II.8.G. 2B. QUERCETIN

Quercetin is one of the most abundant compounds in the flavonol class of flavonoids and their sources include tea, onions, apples and red wine [222]. Quercetin is known to have both anti-oxidant and anti-inflammatory effects. Quercetin is found to possess both hypolipemic and anti-atherogenic properties in experimental animal models [223-225]. The anti-apoptotic effect of quercetin is mediated *via* suppression of the peroxide-induced JNK-c-Jun/AP-1 pathway and the ERK-c-Fos/AP-1 pathway [226]. Quercetin at lower concentrations is found to induce expression of survival and defensive genes, resulting in survival and protective mechanisms whereas higher concentrations activate the caspase pathway, leading to apoptosis [227]. Quercetin and their metabolites reportedly acts *via* PI 3-kinase, Akt/PKB, tyrosine kinases, PKC or MAP kinase signaling cascades [228]. Quercetin inhibits both AP-1 activation and the JNK pathway in TNFα stimulated HUVECs [229] whereas it significantly inhibits TNFα induced E-selectin and ICAM-1 expression in HAECs [210]. The antioxidant activity of quercetin metabolites have been found to protect HG induced injury in HUVECs [230, 231]. Quercetin has inhibitory effects on iNOS and COX-2, as well as on the activation of transcription factors such as NFκB and AP-1 in HUVECs stimulated by a mixture of cytokines [232]. Data are lacking on the anti-inflammatory effects of quercetin on

endothelial cells exposed to HG, including its modulating effects on MCP-1 expression. Therefore, the modulating effects of queretin on HG induced MCP-1 synthesis in aortic endothelial cells were explored.

II.8.G. 3. CURCUMIN

Curcumin is a natural polyphenolic compound abundant in the rhizome of perennial herb turmeric, *Curcuma longa* [233] that has been traditionally used in Indian medicine for over 3000 years now [234]. Curcumin has been shown to have anti-oxidant and anti-inflammatory activity [235]. In cells isolated from diabetic patients, curcumin was found to reduce ROS levels [236]. In experimental studies, curcumin has been shown to reduce hyperlipidemia [237] and in streptozotocin induced diabetic animals, curcumin significantly reduces crosslinking of collagen [238]. Curcumin is also reported to be an inhibitor of NFκB in endothelial cells exposed to different stimuli [197] [239] and only one report suggests that curcumin can inhibit NFκB in HG stimulated endothelial cells [240]. But data is lacking regarding the modulating effect of curcumin on MCP-1 expression in aortic endothelial cells exposed to different stimuli that simulate diabetes. Therefore, the effect of curcumin on HG induced MCP-1 expression in aortic endothelial cells was evaluated.

II.8.G. 4. SODIUM SALICYLATE

Aspirin has become the leading drug in the reduction of cardiovascular mortality followed by beta blockers, statins and ACE-inhibitors. It has a unique place in the treatment of atherosclerosis and its manifestations which result from an interaction between platelet activation, endothelial activation/inflammation and oxidative stress.

After the oral administration of an analgesic dose of aspirin, 50 % is de-acetylated to salicylate immediately after absorption and therefore it is postulated that the anti-inflammatory effects of aspirin are due to the salicylate moiety [241]. NaSal has been shown to inhibit p38 MAPK activity and superoxide generation in human coronary artery endothelial cells [242]. It has also been shown to directly inhibit NFκB activation and consequent expression of pro-inflammatory cytokines and mediators [243]. Sodium salicylate (NaSal) causes uncoupling of oxidative phosporylation thereby decreasing intra-cellular ATP formation, that consequently induce the release of adenosine into the extra-cellular fluids in sufficient quantity to exert anti-inflammatory effects [244]. NaSal is also reported to have inhibitory effects on ox-LDL-mediated LOX-1 and MMP-1 expression in human coronary artery endothelial cells [242]. Data is not available regarding the anti-inflammatory effects of salicylate on endothelial cells exposed to different stimuli that mimic diabetic conditions. Therefore, the effect of NaSal on HG induced MCP-1 expression in aortic endothelial cells was evaluated.

III. MATERIAL AND METHODS

III.1. FINE CHEMICALS

DMEM, BSA, Trypsin, DNase, RNase, HEPES, FBS, EDTA, glucose, monoclonal anti-vimentin antibody, HRP conjugated anti-mouse and anti rabbit IgM antibody, anti-human von Willebrand antibody, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), ECGS, DAPI, FBS, PEG 8000, D-glucose, D-mannitol, curcumin, genistein, sodium salicylate, quercetin, NAC, DEPC, SDS, Tris base, glycine buffer, agarose, coomassie protein assay reagent, sephadex G-25 and sodium acetate were purchased from Sigma-Aldrich, MO, USA. RT buffer, RNase inhibitor, oligo dT, dNTPs, MMLV reverse Transcriptase, GoTaq DNA polymerase, Taq 5Xbuffer and $MgCl_2$ were purchased from Promega Corporation, Madison, WI, USA. Losartan was a kind gift from Dupont, Merck. Minimum essential medium with D-valine (MEM-D-valine) was procured from Pan Biotech, Germany and Bay 11-7082 (3-[(4-methylphenyl) sulphonyl]-2-propenenitril) from Calbiochem, Merck KGaA, Darmstadt, Germany. Trizol reagent, lipofectamine^{TM} 2000 and Opti-MEM was procured from Invitrogen, USA. pGL3 promoter vector and pRL-TK were purchased from Promega Corporation, Madison, WI, USA. Kpn1, Xho-1 and T4 DNA ligase were purchased from New England Biolabs, MA, UK. DiI-Ac-LDL TRITC-labeled anti-rabbit IgG and FITC-labeled anti-goat IgG were purchased from Molecular Probes, Netherlands. SYBR green PCR master mix for real-time PCR was purchased from Applied Biosystems, CA, USA. Non-fat dry milk, LB broth, and LB agar were from Hi Media, Mumbai. Nitrocellulose membrane was from Millipore, USA. Antibodies against p65, p50, c-rel, c-jun, c-fos, fra-1 and IkBα were procured from Santa Cruz Biotechnology, Inc., USA. pMCP-1 Luc plasmids containing the 2.5 kb mouse MCP-1 gene promoter DNA sequence (nucleotides -2466 to +67)

cloned into pGL3 basic luciferase vector was a kind gift from Dr YC Li, Uniiversity of Chicago, USA.

III.2. ROUTINE CHEMICALS

Calcium chloride, disodium hydrogen phosphate, magnesium chloride, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate, sodium hydroxide, chloroform, isopropanol, hydrochloric acid, ethanol and ether were purchased from SISCO Research Laboratories, India.

III.3. COMMERCIALLY AVAILABLE KITS USED

III.3.A. BigDye® Terminator v3.1 Cycle Sequencing Kit for plasmid DNA sequencing was purchased from Applied Biosystems, CA, USA.

III.3.B. Dual Luciferase Reporter Assay system was purchased from Promega Corporation, Madison, WI, USA

III.3.C. ELISA kit for rat MCP-1 was purchased from Biosource International, Inc, Amarillo, CA.

III.3.D. GenElute^{TM} HP midi-prep plasmid DNA isolation kit for plasmid extraction was purchased from Sigma-Aldrich, MO, USA.

III.3.E. NucBuster protein extraction kit for nuclear protein extraction was purchased from Novagen, Merck KGaA, Darmstadt, Germany.

III.3.F. QIAquick PCR Purification Kit for purification of DNA from PCR mix was procured from Qiagen, Germany

III.3.G. QIAquick Gel Extraction Kit for extraction of DNA from gel band was procured from Qiagen, Germany

III.4. CELL CULTURE-WARES

Cell culture-treated 96 and 4 well plates as well as 35 mm, 60 mm and 100 mm dishes were purchased from Nunc, Denmark. Cell culture filter ware was procured from Millipore, USA.

III.5. EQUIPMENTS USED

ELISA microtiter plate reader reader (Bio-Tek instruments, USA), UV-visible spectrophotometer (Shimadzu, Japan), high speed refrigerated centrifuge (Hitachi, Japan), weighing balance (Sartorius, Germany), water bath (LKB, Sweden), ice-flaker (Hoshizaki, Japan), Ph meter (Labindia, India), CO_2 incubator (Nuaire, USA), phase-contrast microscope (Nikon, Japan), laminar flow hood (CLAS, India), magnetic stirrer (Schott, Germany), EASY pure UV/UF compact reagent grade water system (Barnstead, USA), electrophoresis unit (Biorad laboratories, USA), phosphor-imager (Bio-Rad Personal FX,USA), Mini Blot (Biorad laboratories, USA), Programmable thermal cycler (MJ Research Inc, USA), submarine electrophoresis unit (Bangalore Genei, India), InGenius gel documentation system (Syngene Bioimaging,UK) and UV-Transilluminator (Bangalore Genei, India), real-time PCR machine (Applied Biosystems 7300, USA), 3730 DNA Analyzer (Applied Biosystems, USA) and confocal microscope (Leica

□icrosystems, Germany), Sirius single tube luminometer (Berthold Technologies Gmbh & Co., Germany)

III.6. COMPOSITION OF MEDIA, REAGENTS BUFFERS AND REACTION MIXTURES

III.6.1. Acrylamide 30%

Acrylamide- 29.2 % (w/v) and N, N'-methylene bisacrylamide- 0.8% (w/v) in 100 ml deionized water

III.6.2. Agarose gel (1%) for electrophoresis of DNA or RNA samples

Agarose - 200 mg in 20 ml of 0.5X TBE for DNA and RNA

III.6.3. Blocking solution

Skim milk- 2.5% (w/v) and Tween-20- 0.1% (v/v) in 1X PBS

III.6.4. Curcumin stock (10mM)

Curcumin- 3.6838 mg dissolved in 1 ml DMSO

III.6.5. DAB substrate solution

DAB-6mg in 10ml Tris (pH 7.6) containing 10µl of H_2O_2 (30% v/v)

III.6.6. DEPC-treated deionized water

DEPC- 1ml in one liter of deionized water, stirred overnight at room temperature and autoclaved

III.6.7. 2', 7'–dicholrofluorescein diacetate (DCFH-DA) stock (10 mM)

DCHF-DA- 4.8729 mg in 1 ml DMSO

III.6.8. D-Mannitol- 19.5 mM

D-mannitol- 3.55 g in 1 L of DMEM

III.6.9. DNA/RNA gel-loading dye

Bromophenol blue (0.25%; w/v); xylene cyanol FF (0.25%; w/v); EDTA (1mM); glycerol (50%l; v/v) in DEPC-treated deionized water

III.6.10. EDTA (0.5M, pH 8.0)

930 mg EDTA in 5 ml DEPC-treated deionized water

III.6.11. Electrode buffer (pH 8.3) for SDS–polyacrylamide gel electrophoresis (SDS – PAGE)

Tris base- 3.027g/L (25 mM), glycine- 14.4gm/L (192 mM), SDS- 1g/L (0.1%) in deionized water

III.6.12. EMSA binding buffer (5X)

Tris – 50 mM (pH-7.5), NaCl 250 mM, DTT- 5 mM, EDTA- 5 mM, Glycerol 25% (v/v) in deionized H_2O

III.6.13. EMSA buffer (5X)

Tris -30.285 g (pH-8.5), Glycine- 15 g, EDTA- 3.7224 g in deionozed H_2O

III.6.14. EMSA running buffer

120 ml of 5X EMSA buffer is diluted to 600 ml

III.6.15. Ethidium bromide (Stock solution)

EtBr- 10 mg in 1ml deionized water; 1µl of this stock solution was added to 20 ml of 1% agarose gel for DNA/RNA electrophoresis

III.6.16. Gel loading buffer for nucleic acids (6X)

Bromophenol blue -0.25% (w/v), xylene cyanol FF - 0.25% (w/v) and sucrose - 40% (w/v) in dH_2O

III.6.17. Genistein stock (10mM)

Genistein- 2.702 mg dissolved in 1 ml DMSO

III.6.18. Glycerol Stock

Equal volume of plasmid culture and gylecrol-40 % (v/v in autoclaved H_2O) is mixed well and stored at -80°C

III.6.19. Growth medium (pH 7.4)

DMEM containing FBS (20%), benzyl penicillin (100 U/ml) and gentamycin (25 U/ml)

III.6.20. Hank's Balanced Salt Solution without calcium and magnesium (HBSS) (pH 7.4)

Potassium chloride 5.4 mM, potassium dihydrogen phosphate 0.44 mM, sodium chloride 137 mM, disodium hydrogen phosphate 0.63 mM, D-glucose 5.55 mM, sodium bicarbonate 4.17 mM

III.6.21. HG 15 mM

D-glucose- 1.703 g in 1 L of DMEM

III.6.22. HG 25 mM

D-glucose- 3.505 g in 1 L of DMEM

III.6.23. HG 35 mM

D-glucose- 5.306 g in 1 L of DMEM

III.6.24. LB agar

NaCl- 1g, yeast extract- 0.5g, tryptone- 1g, bacteriological agar- 2g in 100ml water and autoclaved before use.

III.6.25. LB broth

NaCl- 1g, yeast extract- 0.5g, tryptone- 1g in 100ml water. Autoclaved before use

III.6.26. Losartan stock (10mM)

4.6201 mg dissolved in 1 ml autoclaved distilled water

III.6.27. Lysis buffer for rat genomic DNA isolation (1 ml)

NaCl (1 M) - 100 µl, SDS (14%)- 71 µl, Tris HCl pH-8 (1 M)- 50 µl, EDTA pH-8 (0.5M)- 200 µl in dH2O- 579 µl

III.6.28. 3-(4-5 dimethyl thiozol-2-yl) 2-5 diphenyl tetrazolium bromide (MTT) stock

MTT- 5 mg in 1 ml of PBS

III.6.29. PAGE 6.6% (25ml)

Acrylamide (30%) - 5.5 ml, EMSA buffer (5x)- 5 ml, APS (10%)- 200 µl, TEMED- 20 µl in deionized H_2O-14.28 ml

III.6.30. Phosphate-buffered saline (pH 7.4)

Sodium chloride (137 mM), potassium chloride (2.7 mM), disodium hydrogen phosphate (10.14 mM), potassium dihydrogen phosphate (1.76 mM)

III.6.31. Ponceau S stain (10X)

Ponceau S – 2g, trichloroacetic acid – 30g, sulfosalicylic acid – 30g were dissolved and made up to 100ml with water. A working solution was prepared by mixing 1 part of the 10X stock with 9 parts of water.

III.6.32. Quercetin stock (10mM)

Quercetin- 3.383 mg dissolved in 1 ml DMSO

III.6.33. Resolving Gel for SDS–PAGE (10%) - 10 ml

Acrylamide- 30% (3.3 ml), Tris- 1M (pH 6.8; 2.5 ml), 10% SDS- 0.15 ml, 10% APS- 0.1 ml and TEMED-0.004 ml were added to 3.946 ml of deionized water

III.6.34. Serum-free medium

DMEM containing antibiotics penicillin (100 U/ml) and gentamycin (25 U/ml)

III.6.35. SDS gel-loading buffer (1X)- 10 ml

SDS- 3 ml (20% w/v), bromophenol blue 0.006 g, β-mercaptoethanol 1.6 ml, glycerol- 3 ml in 2.4 ml Tris – 1 M (pH 6.8)

III.6.36. Sodium acetate (3M, pH 5-6)

Sodium acetate- 23 g in 5 ml DEPC-treated deionized water

III.6.37. Sodium salicylate stock (10mM)

Sodium salicylate- 1.601 mg dissolved in 1 ml autoclaved distilled water

III.6.38. Solution I - GTE

Glucose- 50mM, EDTA- 10mM, Tris Cl- 25mM (pH 8), RNaseA- 50 μg/ml

III.6.39. Solution II- Lysis

SDS- 1%, NaOH- 0.2 N

III.6.40. Solution III- Neutralization

5M Potassium Acetate- 60ml, acetic acid- 11.5 ml, nuclease free water- 28.5 ml

III.6.41. Stacking gel for SDS – PAGE (5%)

Acrylamide- 30% (0.35 ml), 1M Tris- 0.25 ml (pH 6.8), SDS- 10% (0.02 ml), APS- 10% (0.02 ml) and TEMED- 200 µl were added to 1.4 ml of deionized water

III.6.42. Towbin's buffer (Transfer buffer)

Trisma base- 3.027 g, glycine- 14.4 g, methanol- 200 ml made up to 1 L with deionized water

III.6.43. Tris borate EDTA buffer (TBE) (5X, pH 8.3)

Tris base- 54g; boric acid- 27.5g; EDTA- 20ml (0.5M, pH 8.0) made up to 1L with deionized water

III.6.44. Tris-buffered saline (10X, pH 7.6)

Tris base- 24.2 g, sodium chloride- 80 g in 1L deionized water

III.6.45. Tris-buffered saline with Tween-20 (TBST) [1X]

1X TBS containing 0.1% Tween-20

III.6.46. Tris-CaCl2 buffer (4X, pH 7.4)

Tris (200mM), $CaCl_2$ (200mM)

III.6.47. Tris-EDTA buffer (pH8.0)

TrisCl- 10mM, EDTA- 1mM

III.6.48. Tris glacial acetic acid and EDTA buffer (TAE) (50X)

Tris base- 242 g, 0.5M EDTA- 100 ml, glacial acetic acid- 57.1ml in 1 L deionized water

III.6.49. Trypsin-EDTA solution

Trypsin (0.25% w/v) and EDTA (0.2% w/v) in PBS (pH 7.4)

III.6.50. cDNA mix (30 µl)

5X RT buffer	6 µl
dNTPs	2.5 µl
Oligo dT	3 µl
RNAse inhibitor	0.5 µl
M-MLV RT	2 µl
Total RNA (1 or 5 µg)	10 µl
DEPC treated water	6 µl

III.6.51. Semiquantitative PCR mix (25µl)

H_2O	13.5µl
GoTaq buffer (5X)	5 µl
$MgCl_2$	1.75 µl
dNTPs	1.5 µl
Forward primer (5pmol)	1 µl
Reverse primer (5pmol)	1 µl
GoTaq	0.25 µl
cDNA	1µl

III.6.52. Real-Time PCR mix (15 µl)

H_2O	6
SYBR Green mix (2X)	7.5 µl
Forward primer (1.25pmol)	1 µl
Reverse primer (1.25pmol)	1 µl
cDNA	1µl

III.6.53. High fidelity PCR mix for promoter sequence cloning (20 µl)

Master mix 1(10 µl)

Triple master mix	0.3 µl
Hugh fidelity buffer (10X)	2 µl
dNTPs	1 µl
H_2O	6.7µl

Master mix 2 (10 µl)

Genomic DNA (100ng)	5 µl
Forward primer (5pmol)	1 µl
Reverse primer (5pmol)	1 µl
H_2O	3 µl

III.6.54. ^{32}P-end labeling mix for NFκB or AP-1 oligonucleotide (12.5 µl)

dd H_20	4.75 µl
T4 Kinase buffer (10x)	1.25 µl
Oligo- 10pmol (AP-1 or NFκB)	2 µl
T4 kinase (10u)	1 µl

III.6.55. EMSA DNA- oligonucleotide binding mix (10 µl)

dd H_20	5 µl
5X binding buffer	2 µl
Protein	2 µl
^{32}P-labeled oligos (AP-1 or NFκB)	1 µl

III.6.56.Plasmid DNA double digestion mix (40 µl)

Plasmid DNA (2.5 µg)	4 µl
Nuclease free H_20	27 µl
NEB buffer II (10X)	4 µl
BSA (10X)	4 µl
Kpn1	0.5 µl
Xho1	0.5 µl

III.6.57. Plasmid DNA ligation mix (10 µl)

NFκB or AP-1 insert (100 ng)	1 µl
pGL3 promoter vector (25 ng)	1 µl
Ligation buffer (2X)	5 µl
DNA ligase	1 µl
DNA dilution buffer (5X)	2 µl

III.6.58. Plasmid DNA Sequencing mix

Master Mix 1

Plasmid DNA (100-200 ng)	1 µl
GL2 or RV3 Primer (2.5 pmol)	1 µl
Nuclease free H_20	1.2 µl

Master Mix 2

Big Dye + Buffer (1:7)	1.8 µl

III.7. ISOLATION, CULTURE AND CHARACTERIZATION OF AORTIC ENDOTHELIAL CELLS FROM RATS (RAECs)

III.7. A. Isolation of RAECs

Animal care and use were approved by the Institutional Animal Ethics Committee and conformed to APS guidelines.

RAECs were isolated from male Sprague-Dawley rats following a previously reported method with modification [245]. Male Sprague-Dawley rats 2-3 months old were anaesthetized using diethyl ether. The heart and lungs were aseptically removed from the chest cavity and placed in a petri dish containing Hank's balanced salt solution (HBSS). The lungs were dissected away by cutting the pulmonary vasculature. The aorta was carefully removed from the heart, the connective tissue surrounding the exterior of the vessel teased away and the vessel washed briefly in HBSS **(Figure 10)**.The aorta was cut into rings using a sterile scalpel blade. The resulting aortic rings were placed on edges onto the 2% gelatin coated 35mm tissue culture dish and allowed to anchor to the bottom for 3-5 minutes **(Figure 11).**

Figure 10: Dissected rat aorta after removing the connective tissue

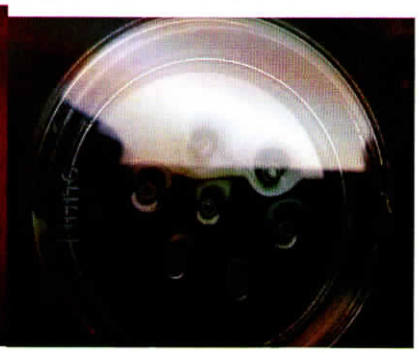

Figure 11: The aortic rings placed on edges onto the cell culture dish

DMEM containing 20% heat inactivated FBS, ECGS (100ug/ml), penicillin (100 U/ml) and gentamycin (25 U/ml) was carefully added along the sides of the dish so as not to dislodge the tissue explant. The medium was carefully changed every 2 days. Endothelial cells started migrating from the edges of the explant after 2 days of culture initiation. When there were enough cells, the aortic rings were carefully removed using a sterile forceps and 2 ml of medium was added and cells allowed to grow to confluence.

III.7. B. Sub-culture of RAECs

At confluence, the cells were split at 1:3 ratio. Cells were washed thrice with PBS and trypsinized using Trypsin (0.25%) – EDTA (0.2%) in PBS and the detached cells were collected immediately in 15 ml centrifuge tubes containing equal volume DMEM with 20% FBS. More PBS was added to the trypsin treated culture dish and pipetted repeatedly without foaming to dislodge remaining cells from the dish. The suspension was centrifuged and the pellet was resuspended in complete medium.

To avoid possible fibroblast and vascular smooth muscle cell contaminations, the RAECs were cultured in MEM with D-valine containing 20% FBS for 10-15 days immediately after 1st passage.

III.7. C. Characterization of RAECs

RAECs were characterized by their morphology, immunocytochemistry and growth kinetics.

III.7. C.1. Morphology

The cells were routinely viewed under an inverted phase contrast microscope to analyze the cobblestone morphology which is a typical characteristic feature of endothelial cells.

III.7. C.2. Immunocytochemistry

Cells grown on gelatin-coated culture dishes were washed thrice with PBS and fixed in ice-cold acetone-methanol 1:1 (v/v) for 7 minutes. After two washings in PBS, endogenous peroxidase was quenched with two drops of 3% hydrogen peroxide for 5 minutes. Cells were again washed with PBS. Nonspecific binding was blocked with 3% BSA in PBS for 10 minutes. Cell were then incubated with primary antibody against human von Willebrand factor (1:800) or vimentin (1:50) in PBS containing 1% BSA for 2 h. The cells were washed thrice with PBS and incubated for 30 minutes with diluted secondary anti-mouse IgG antibody (1:50) conjugated with either HRP or alkaline phosphatase. Cells were washed thrice with PBS and incubated with either the substrate reagent containing 3-amino, 9- ethyl-carbazole or the substrate solution for alkaline phosphatase for up to 10 minutes and observed under a microscope. After sufficient color development, the cells were washed in deionized water for 5 minutes. The cells were counterstained with hematoxylin, mounted in glycerol and observed under a microscope.

III.7.C.3. Uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate labeled Acetylated Low Density Lipoprotein (DiI-Acetylated LDL)by RAECs

RAECs grown on gelatin coated glass cover slips were used for testing the scavenger pathway of lipoprotein metabolism according to a previously reported procedure [246]. Cells were incubated with 10μg/ml DiI-Ac-LDL at 37°C, in growth medium containing 20% FBS, for 4 h. The media was then removed and the cells were washed once with probe-free media for 10 minutes, rinsed with PBS, and fixed with paraformaldehyde for 5

min. Cover slips were inverted over a drop of 10% PBS in glycerol prior to viewing under a fluorescent microscope or confocal microscope.

III.8. DESIGN OF THE STUDY

The cells were used for the experiments during passages 3-7. In all experiments, the cells were used at near full confluence except those involving quercetin where confluent cultures were used including its control group. RAECs were rendered quiescent by serum starving for 48 h in DMEM supplemented with 0.4% heat-inactivated FBS. RAECs were then treated for 24 to 72 h with DMEM containing 1% FBS at four different of D-glucose concentrations as follows: 5.5mM/L (control), 15mM/L, 25mM/L or 35mM/L D-glucose (high glucose concentrations) or with an osmotic control media (5.5 mM of D-glucose plus 19.5 mM of L-glucose). For inhibitory studies, RAECs were exposed to HG concentration of 25 mM in the presence or absence of curcumin (1-30 μM), NFκB inhibitor- Bay11-0782 (5 μM), quercetin (1-100 μM), NAC (10 mM), genistein (1-50 μM), sodium salicylate (1-100 μM) or losartan (1-100 μM). EA.hy 926 cells were used for transient transfection and luciferase reporter assay. At the end of treatments, the following methods were used for the studies.

III.9. GROWTH KINETICS OF RAECs

Growth kinetics of RAECs was studied as reported previously [247]. RAECs from passage 3 were plated in 35 mm cell culture dishes at a density of 1×10^5 cells/ml and exposed to different glucose concentrations. Cell count was determined using a Neubauer chamber at 0, 24, 48, 72 and 96 h after seeding. A graph was plotted with cell number on

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the y-axis and time on the x-axis. The best fitting curve was constructed by connecting the mean points. A time interval was selected in the middle of the exponential phase where the relationship between time and log density is linear. The beginning of the time interval was marked as t_1 and the end as t_2. The time interval, $t_2 - t_1$ was taken as Δt. The cell densities at t_1 and t_2 were taken as N_1 and N_2 respectively. The growth constant, k, was calculated using the formula:

$k = [2.3 \log (N_2 / N_1)] / \Delta t \text{ hours}^{-1}$.

The doubling time, T_d, was determined using the formula:

$T_d = 0.693/k \text{ h}$

III.10. 3-(4-5 DIMETHYL THIOZOL-2-yl) 2-5 DIPHENYL TETRAZOLIUM BROMIDE (MTT) ASSAY

The viability of cells subjected to different treatments were analyzed using MTT assay as previously reported [248]. Cells were seeded at a density of 5000 cells/ well in 96 well microtiter plates and incubated in medium containing different glucose concentrations for 24, 48 and 72 h. After the incubation period, the medium from each well was removed, fresh medium with 20 µl of MTT (5 mg/ml stock) added and kept in CO_2 incubator till formazan crystals were formed (2 ½ h). Formazan crystals were later solubilized using acidic iso-propanol and the optical density at 570 nm was measured using a microtiter plate reader with the extraction buffer as a blank. The OD in control group was taken as 100% of viability. The relative cell viability in percentage was calculated using the formula

$$\frac{A_{570} \text{ of treated samples}}{A_{570} \text{ of untreated samples}} \times 100$$

III.11. MCP-1 ELISA

RAECs were exposed to various glucose concentrations for 24 to 72 h in the presence or absence of different concentrations of specified molecules. The cell culture supernatants were collected centrifuged at high speed for 20 seconds to remove possible cell debris and the clear supernatants stored at –80°C until assay. To quantify MCP-1 protein secretion in the media, a commercial solid phase quantitative sandwich ELISA kit specific for rat MCP-1 with sensitivity of 8 pg/mL was used.Assays were performed according to manufacturer's protocol.

III.12. RNA ISOLATION, cDNA SYNTHESIS AND PCR

III.12.A. RNA isolation

Total RNA was isolated from RAECs subjected to different treatments by an improvement of the single-step method reported previously [249] using Trizol reagent as per the instructions of the manufacturer. The purity of RNA was determined from the ratio of absorbance at A_{260}/A_{280} ratio. The yield of RNA was calculated from the formula

$$\text{RNA in } \mu g/\mu l = \frac{A_{260} \times 40 \times DF}{1000}$$

Where, A_{260} is the absorbance at 260 nm, DF is the dilution factor and 40 is included assuming that 1 A_{260} unit ≡ 40 µg/µl of RNA

Total RNA was isolated using TRIZOL reagent from cells exposed to different treatment conditions. Briefly, isolated RNA (5μg) was reverse transcribed using MMLV-RT to obtain the cDNA.

III.12.B. cDNA Synthesis

Total RNA in DEPC-treated water and oligo dT primers were mixed and heated at 70 ^{o}C for 5 min. The heated mixture was immediately cooled on ice. To this mixture, 5X RT buffer, dNTPs, RNAse inhibitor, MMLV reverse transcriptase and DEPC-treated water were added. The reaction mix was incubated at 37 ^{o}C for 2 hours, heated for 5 minutes at 90 ^{o}C to inactivate MMLV-RT and rapidly cooled on ice. The cDNA preparations were stored at -20 ^{o}C until use.

III.12.C. Semi-quantitive RT-PCR

cDNA synthesized from 5 μg RNA was amplified by PCR using previously published gene specific primers for GAPDH and MCP-1.[250] The primer sequences used were as follows: MCP-1 sense primer, 5' TGT TGT TCA CAG TTG CTG CCT G 3'; MCP-1 antisense primer 5' GTG CTG AAG TCC TTA GGG TTG AT 3': GAPDH sense primer 5'.CCC TCA AGA TTG TCA GCA ATG C 3'; GAPDH antisense primer 5' GTC CTC ATG TTA GCC CAG GAT 3'. PCR settings for MCP-1 and GAPDH were as follows: Initial denaturation for 30s at 95°C followed by 30 cycles of amplification for 15s at 95°C, 30s at 61°C and 1 min at 72°C followed by final extension for 7 min at 72°C. The PCR products were then resolved on 1% agarose gel containing ethidium bromide and visualized using UV transilluminator. The images were captured with gel documentation system and the resultant bands were quantified using densitometry with Gene tools

software (Syngene Bioimaging, UK).Results are expressed as fold stimulation over control after normalizing with paired GAPDH mRNA levels.

III.12.D. Real-time RT-PCR

Total RNA from cells subjected to different treatment conditions was isolated using TRIZOL reagent (Invitrogen) and 1 μg of total RNA was reverse transcribed using MMLV-RT and oligo (dT) primers (Promega). The cDNA was then amplified by PCR using gene specific primers for GAPDH and MCP-1 [250]. The primer sequences used were as follows: MCP-1 sense primer 5' TGT TGT TCA CAG TTG CTG CCT G 3'; MCP-1 antisense primer 5' GTG CTG AAG TCC TTA GGG TTG AT 3'; GAPDH sense primer 5' CCC TCA AGA TTG TCA GCA ATG C 3'; GAPDH antisense primer 5' GTC CTC ATG TTA GCC CAG GAT 3'. Real- time PCR was performed using a SYBR green PCR reagent kit in real time PCR machine. PCR settings were as follows: Initial denaturation for 30s at 95°C followed by 40 cycle of amplification for 15s at 95°C, 30s at 62°C and 1 min at 72°C with subsequent melting curve analysis increasing the temperature from 60°C to 95°C. Dissociation curves were used to verify the amplicons. The relative amount of MCP-1 mRNA was normalized to GAPDH mRNA levels and the changes in mRNA levels were determined using the formula $2^{-\Delta\Delta Ct}$.

III.13. EXTRACTION OF NUCLEAR PROTEIN AND EMSA

III.13.A. Preparation of nuclear extract

Nuclear extracts were prepared from RAECs exposed to different treatment conditions using NucBuster protein extraction kit (Novagen) following manufacturer's instructions

and stored in aliquots at -80°C until assay. Protein concentrations of the nuclear extracts were determined by Bradford assay.

III.13.B. Bradford protein assay [251]

Briefly, 2.5 µl of lysates were diluted in 122.5 µl of deionized H_20 and mixed well. 50 µl of each sample in duplicates were mixed with 200 µl of coomassie protein assay reagent (diluted 1:5 in deionized H_20), incubated at room temperature in dark for 10 min and spectrophotometric readings taken at 570 nm using microtitre plate ELISA reader. Deionized H_20 (50 µl) with 200 µl coomassie protein assay reagent (diluted 1:5) was used as blank. The concentration of protein in the lysates was calculated using the following formula:

(Test- blank) X 6 = 'x' µg/ µl

III.13.C. ^{32} P-labeling of NFκB and AP-1 oligonucleotides

Reaction mixtures containing single stranded NFκB consensus oligonucleotide from the human immunodeficiency virus-1 long terminal repeat 5'-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3' containing the κB site (underlined) [252] or AP-1 consensus oligonucleotide 5'-CGCTTGATGACTCAGCCGGAA- 3' containing AP-1 binding site (underlined) [253] were incubated in separate reaction tubes along with γ^{32} P and T4 polynucleotide kinase and incubated at 37°C for 45 minutes. The reaction was stopped by adding 0.3 µl of 0.5 M EDTA and loaded onto G-25 sephadex columns (pre-washed thrice with TE buffer, pH 8.0) and centrifuged at 1600 rpm for 4 minutes at RT. 50 µl deionized H_2O was added to column and the above step was repeated . γ^{32} P activity was assayed in 3 µl from each sample using liquid scintillation spectrometry. Afterwards, 100 fold molar excess of 3'

AP-1 or 3' NFκB oligo (antisene) was added to the 5' γ^{32} P labeled AP-1 or NFκB oligo respectively, denatured for 3 minutes in boiling waterbath and kept for annealing at RT for 2 h. The mix was then aliquoted to 75,000 counts per 4 µl and stored at -80^{0}C until use.

III.13.D. Nuclear protein- DNA binding reaction and supershift analysis

Nuclear extracts (10 µg) were incubated with γ^{32}P-end labeled double stranded NFκB or AP-1 oligonucleotide at 37 °C for 30 min in the presence of 1 µg/ml poly(Di-Dc) in a binding buffer (25 Mm HEPES (Ph 7.9), 50 Mm NaCl, 0.5 Mm EDTA, 0.5 Mm DTT, 1% Nonidet P-40, and 5% glycerol). After the initial incubation period for protein-DNA interaction, 3 µg of each antibody was added to binding reaction and further incubated for 30 minutes at 37°C. NFκB bands were supershifted using antibodies to p65, p50 and c-rel whereas AP-1 bands were supershifted with antibodies to c-jun, c-fos and fra-1. The DNA-protein complex was resolved using a 6.6% native polyacrylamide gel at 150V for 90 minutes. The gels were dried and the radioactive bands were visualized by phosphorimaging (Bio-Rad Personal FX)[254]. The specificity of the protein-DNA interaction was confirmed by competition with 100-fold molar excess of unlabelled cold NFκB or AP-1 oligonucleotide probe to the binding reaction.

III.14.CELL IMAGING USING CONFOCAL MICROSCOPY

The nuclear accumulation of NFκB sub-unit, p65 and AP-1 subunit, c-jun in HG stimulated RAECs was studied using confocal microscopy. Cells grown on gelatin-coated glass cover slips (>70% confluence) were subjected to different treatments and fixed in ice-cold acetone-methanol 1:1 (v/v) for 7 minutes. After two washings in PBS,

nonspecific binding was blocked with 3% BSA in PBS for 10 minutes. The cells were then incubated with antibody against p65 diluted 1:500 in PBS containing 1% BSA for 2 h at 37°C. The cells were washed thrice with PBS and incubated for 30 minutes with FITC- labelled anti-goat (1:100 dilution) or TRITC- labelled anti-rabbit IgG (1:100 dilution) in dark. The nucleus was counterstained with DAPI. The cells were washed with PBS, mounted in glycerol and viewed under confocal microscope. Fluorescence emission was detected at 520 nm with an excitation wavelength of 490 nm.

III.15. DETERMINATION OF INTRACELLULAR ROS USING 2',7'–DICHOLROFLUORESCEIN DIACETATE (DCFH-DA)

Quiscent RAECs were incubated in 10 μM DCFH-DA for 45 minutes. The cells were stimulated with HG for 2 h in the presence or absence of NAC (100 μM) or Quercetin (100 μM). DCF fluorescence emission was detected at 535 nm with an excitation wavelength of 485 nm. Imaging of DCF emission was taken using fluorescence microscope.

III.16. WESTERN BLOT ANALYSIS FOR CELLULAR PROTEIN DETERMINATION

III.16.A. Protein extraction

Western blot analysis was carried out as described previously[255] with minor modifications. Briefly, RAEC cultures were exposed to normal (5.5 mM) or higher glucose (25 mM) concentrations in the presence or absence of quercetin or NAC and western blot analysis was done for p65, IκBα and β-actin proteins. The total cellular protein or nuclear protein was extracted using CelLytic^{TM} MT Mammalian tissue

lysis/extraction reagent or Nuc-Buster protein extraction kit respectively as per manufacturer's instructions. The protein content of the lysate was determined using the Bradford assay.

III.16.B. SDS-PAGE

The 40 µg of protein from each sample was mixed with SDS gel-loading buffer and incubated in boiling water bath for 10 minutes and there after rapidly cooled on ice. The samples were then loaded into each well of the 10 % SDS-PAGE minigel gel. Appropriate molecular weight marker was loaded into one of the wells. The gel was run at constant voltage of 70 V until the dye front reached the level of the resolving gel. Once the dye front entered resolving gel the voltage was increased to 100 V.

III.16.C. Electrotransfer

The nitrocellulose membrane, filter, and absorbent pads were soaked in Towbin' transfer buffer for at least 10 minutes. The resolved gel was removed from electrophoresis apparatus and the stacking gel was cut away. The proteins resolved in the gel were elctotransfered to nitrocellulose membrane in Towbin' transfer buffer using the transfer apparatus at 100 V for 2 h. At the end of transfer, the membrane was stained with ponceau S to ensure successful transfer of protein bands.

III.16.D. Immunodetection

The membrane was washed twice with PBS-T for 5 minutes and blocked for 1 h with 5% skim milk. The membrane was then incubated overnight at 4^0C with the primary antibody prepared at a dilution of 1:200 in PBS-T containing 5% BSA. Unbound primary antibody was removed by washing (3 X 5' times) with PBS-T and the membrane was incubated for 1 h with HRP-conjugated anti-mouse/anti-goat secondary antibody diluted 1:1000 in

PBS-T containing 5% BSA. The unbound secondary antibody was removed by washing the membrane (3 X 5' times) with PBS-T. The membrane was incubated with DAB till the colour developed, washed in deionized H_2O, dried and the images were captured using gel documentation system.

III.17. CLONING OF AP-1 AND NFκB SEQUENCES UPSTREAM OF RAT MCP-1 PROMOTER INTO pGL3 REPORTER VECTOR

The pNFκB Luc plasmid containing the NFκB binding sequence (-2407 to -2065) and the pAP-1 Luc plasmid containing the AP-1 binding sequence (-241 to -95) in the rat MCP-1 promoter was constructed by cloning these sequences separately into pGL3 promoter vector. pMCP-1 Luc plasmids containing the 2.5 kb mouse MCP-1 gene promoter DNA sequences (nucleotides -2466 to +67)cloned into pGL3 basic luciferase vector was used for promoter activation studies whereas pNFκB Luc plasmid and pAP-1 Luc plasmid were used for enhancer activation studies (**Figure 12**). The pRL-TK plasmid vector was used as an internal control along with all the above three constructs.

GTAGAGGCTCAATCCTCACCCCCTTATATCTCTTTTCTGGGCCTTTCCCTTGGCTTTCCAAGTCAGAGCTCAGACTATGC
CTTTGTTGAGCTATTCCAGATTCTCAGGCCCTTGTGAGAGCTGCTTGGCTGTAAGCCCAGCATCTGGAGCTCATATTCCA
GCTAAATATCTCTCCTGAAGGGTCTGGGAACTTCCAATACTGCCTCAGAATGGGAATTTCCACACTCTTATC
κB (A) (-2281 to -2272) **κB (B)** (-2255 to -2248)
CTACTCTGCCTCTGACCTACCAGCTGGGAAGAGCATCCTTTGTTGACAGAGTAAAGTGAGTGGGAGAGAGACAATTATT
TTCCTTTCTTTCGTTTATGATTCATACTGTGTTGCA.....
.....
.....TCGTATGCTAACTGAAGCTTGCAGTGCCAATT
CACTTTCCAATGGTGACCTCCCAGGCGGTTTCTCCCTTCTACTTCCTGGAAACATCCAAGGGCTCGGCACTTACTCAGCA
GATTCAAACTTCCACTTTCCATCACTCATCGAGGATGATGCTGCTCCTTGGCACCAACCACCCTGCCTGACTCCAGCCTC
AP-1(-54 to -44)
TGGCTTACAATAAAAGGCTGAGGCAGAGCCGCTAGAAAT(+1)GCAGAGACACAGACAGAGGCCAGCCCAGAAACCAG
CCAACTCTCACTGAAGCCAGATCTCTCTTCCT(+67)CCACCACTATG

Figure 12: The 2.533 kb MCP-1 gene promoter DNA sequence corresponding to nucleotides -2466 to +67 which includes the NFκB and AP-1 enhancer seuences. The nucleotides are numbered based on the sequence of rat MCP-1 gene promoter. The two sequences highlighted in yellow correspond to the two NFκB binding sites- site A and site B. The sequences highlighted in blue correspond to the AP-1 site.

III.17.A. Genomic DNA isolation from rat tail biopsies and PCR for promoter sequence cloning

III.17.A.1. Isolation of genomic DNA from rat tail

Lysis buffer (500 µl) containing proteinase K (0.25 mg) was added to rat tail (0.5 cm) in a 1.5 ml eppendorf tube and incubated overnight at 55 °C with gentle shaking. 0.7 ml of equilibrated phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed vigorously and centrifuged at 13,000 rpm for 5 minutes at RT. The upper aqueous phase was transferred to a new microfuge tube. The aqueous phase was gently drawn through the 1 ml tip several times after transfer. 1 ml of 100% ethanol (ice cold) was added and mixed gently to precipitate the DNA. The tube was now centrifuged at 13,000 rpm for 5 minutes and the supernatant was carefully removed. 1 ml of 70% ethanol was added and inverted several times and centrifuged at 13,000 rpm for 5 minutes. The supernatant was carefully discarded and the pellet was air dried at RT. 100-200 µl of TE buffer was added and incubated at 65 °C for 10- 15 minutes to resuspend the genomic DNA. The DNA was gently drawn through P1000 tip several times after 65 °C incubation to aid in suspension. The total yield was approximately 20-50 µg DNA (0.1-0.25 µg/µl).

III.17.A.2. Primer design and high fidelity PCR for promoter sequence cloning

Primers were designed with the Kpn I **(5'GGTACC 3')** and Xho I **(5' CTCGAG 3')** sites on the 5' ends using Amplifx 1.37 software. AP-1 and NFκB consensus binding sequences upstream of MCP-1 gene promoter were amplified by PCR from rat genomic DNA using the high fidelity Triple Master polymerase mix. The following sets of primers were used:

AP-1 F - 5' GATACA**GGTACC**GACACTTGTGGTCACAGTCTAGCA 3'

AP-1 R - 5' GATAC**CTCGAG**GTGGAGTCAGGCAGGGTGGTT 3'

NFκB F - 5' GATACA**GGTACC**GGACCAGTAGAGGCTCAATCCTCA3'

NFκB R - 5' GATAC**CTCGAG**GGGAGGTCACCATTGGAAAGTGAA 3'

PCR settings for AP-1 site were as follows: Initial denaturation for 30s at 95°C followed by 35 cycle of amplification for 30s at 95°C, 30s at 64.5°C and 1 min at 72°C followed by final extension for 7 min at 72°C.

PCR settings for NFκB site were as follows: Initial denaturation for 30s at 95°C followed by 35 cycle of amplification for 30s at 95°C, 30s at 55°C and 1 min at 72°C followed by final extension for 7 min at 72°C.

III.17.A.3. PCR product purification

The PCR amplified AP-1 and NFκB sequences were purified using QIAquick PCR purification kit to remove traces of buffer and polymerase enzyme according to manufacturer's instructions.

III.17.B. Restriction digestion of pGL3 basic vector, AP-1 and NFκB inserts

Kpn I and Xho I restriction enzymes were used to digest the pGL3 promoter vector and the purified PCR products of AP-1 and the NFκB inserts. The double digestion using restriction enzymes were carried out in NEB buffer II for a period of 4-6 h at 37°C water bath. The pGL3 promoter vector has Kpn I and Xho I restriction sites at 5 and 32 bp respectively **(Figure 13).**

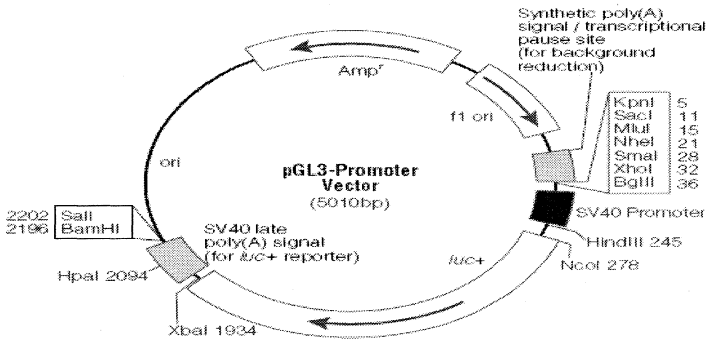


Figure 13: The pGL3-promoter vector circle map. Additional description: *luc*+, cDNA encoding the modified firefly luciferase; Ampr, gene conferring ampicillin resistance in *E. coli*; f1 ori, origin of replication derived from filamentous phage; ori, origin of plasmid replication in *E. coli*. Arrows within *luc*+ and the Ampr gene indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis.

III.17.B.1. Gel band purification of restriction digested products

The restriction digested plasmid as well as the AP-1 and NFκB inserts were run on 1% agarose gel and the bands of interest of correct size were rapidly cut out from the gel under UV transilluminator. The gel pieces were purified using QIAquick column gel extraction kit according to manufacturer's instructoins.

III.17.C. Ligation of the vector and insert

T4 DNA Ligase enzyme from NEκB was used in ligating the sticky ends of pGL3 vector with that of either the AP-1 or NFκB inserts. The ligation reaction was set for 16 h at 18°C in the ligation buffer.

The amount of insert to be used for ligation was calculated using the following equation:

$$\textbf{Amount of insert} = \frac{\textbf{Insert size} \times \textbf{Conc. of vector}}{\textbf{Vector size}}$$

III.17.D. Transformation and screening of colonies

Ultra competent cells of DH5α E. coli strain were thawed on ice and 10 µl of the ligated product was added to it and incubated on ice for 20 minutes. Heat shock treatment was given for 60 s at 42°C, following which they were kept on ice for 5min. Later, 800 µl of LB broth was added and incubated at 37°C for another 1 h. The inoculum was plated in LB agar amp plates and incubated at 37°C for 12 h for ampicillin resistant colonies to grow.

III.17.E. Plasmid mini prep

The ampicillin resistant colonies that grew, were picked and inoculated into 3 ml LB broth containing ampicillin (100 µg/ml) for 12 h at 37°C. A glycerol stock of each colony was prepared and stored in -80°C.The remaining cells were pelleted by centrifuging at 3000 rpm for 5 minutes. After completely removing the medium, the pellet was resuspended in GTE (solution I). The cells were lysed by adding solution II to solution I by gentle inversion and were finally neutralized by solution III. The lysates were centrifuged at 13000 rpm at 4°C for 15 minutes and the supernatant excluding the precipitate was carefully removed to fresh microfuge tube. Equal volumes of phenol (equilibrated in Tris Cl pH.8) and chloroform: isoamylalcohol (24:1) was added, mixed well and centrifuged at 13000rpm at room temperature for 5 minutes. After centrifugation, the upper aqueous layer was transferred to a fresh microfuge tube and two volumes of absolute ethanol was added and incubated at -70°C for 30 minutes and centrifuged at 13000 rpm at 4°C for 20 minutes. The pellet was washed with 70% ethanol

and centrifuged at 13000 rpm for 10 minutes. After draining off the supernatant, the pellet was air dried and resuspended in TE buffer or nuclease free water.

III.17.F. DNA sequencing and analysis for positive clones

The plasmid DNA that was isolated from colonies after the transformation of ligated pGL3 promoter vector with AP-1 or NFκB inserts were subjected to DNA sequencing. For this purpose, PCR was done using BigDye® Terminator v3.1 Cycle Sequencing Kit with pGL3 promoter vector specific forward primers and the above isolated plasmid DNAs in sequencing plates. PCR settings were as follows: Initial denaturation for 3 min at 94°C followed by 30 cycle of amplification for 10s at 94°C, 10s at 55°C and 4 min at 60°C. The following pGL3 promoter vector specific forward primers were used:

GL2 primer **5' GAAATACAAAAACCGCAGAAGG 3'**

RV3 primer **5' CTAGCAAAATAGGCTGTCCC 3'**

III.17.F.1. Processing of samples for sequencing

After PCR, absolute ethanol (25 µl) and 3M sodium acetate (pH 5.2; 1 µl) was added to each sample in the sequencing plate and incubated for 5 minutes at RT. The sample was centrifuged at 4000 rpm for 20 minutes at 20°C.The supernatant was removed and 80% ethanol (100 µl) was added and centrifuged at 4000 rpm for 20 minutes at 20°C. The supernatant was removed and the pellet was air dried. Before sequencing 50% Hidi formamide was added to each well and the sequencing of the PCR products was done using 3730 DNA Analyzer.

DNA sequence data (chromatograms) were converted to nucleotide sequences using GENETOOL software and the sequences were analyzed by matching it with AP-1 and NFκB consensus binding sites in the rat MCP-1 promoter sequences using the BLAST (bl2seq) programme available in http://blast.ncbi.nlm.nih.gov/Blast.cgi

III.17.G. Preparation of transfection grade plasmid

Transfection grade plasmid was prepared using the GenElute^{TM} HP midi-prep plasmid DNA isolation kit according to manufacturer's instruction or by PEG purification.[256]

III.17.G.1. PEG purification

The glycerol stock of pMCP-1 luc, pAP-1 Luc and pNFκB Luc were inoculated separately into 3 ml LB Amp media. After 6 h, 1 ml from the culture was inoculated into 100 ml of LB Amp media and grown further at 37°C for 12 h. The cells were pelleted and resuspended in 7.5 ml of ice cold GTE by vortexing, lysed using solution II (15 ml) and neutralized with solution III (10 ml). This was spun at 12000 rpm for 15-30 minutes and the supernatant was transferred to fresh centrifuge tubes. To this, isopropanol (0.6 volumes) was added, mixed and kept at room temperature for 10 minutes and then centrifuged at 12000 rpm for 15 minutes at 4°C. The pellet was dissolved in 500 µl TE (pH 8). Equal volume of 5M LiCl was added, mixed and centrifuged at 4°C at 10000 rpm for 10 minutes to precipitate the RNA. The supernatant was transferred to a fresh microfuge tube and equal volumes of isopropanol was added and centrifuged at 10000 rpm for 10 minutes at room temperature. The pellet was washed with 70% ethanol and centrifuged at 10000 rpm for 10 minutes at 4°C. The pellet was dissolved in TE (500 µl)

containing RNase (20 μg/ml) and incubated at 37°C for 30 minutes. To this, 1.6 M NaCl containing 13% PEG 8000 was added, mixed and kept on ice for 1 h and centrifuged at 12000 rpm for 5 minutes at 4°C. The pellet was redissolved in TE and extracted with Phenol: Chloroform: Isoamylalcohol (25:24:1). The supernatant was transferred into a fresh tube to which ethanol (twice the volume of supernatant) and 10 M Ammonium acetate ($1/10^{th}$ volume) were added, mixed and incubated at room temperature for 10 minutes. Later the solution was centrifuged at 13000 rpm for 5 minutes at 4°C and the pellet was washed with 70% ethanol and centrifuged at 13000 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet was air dried and suspended in TE buffer (pH- 7.4) for use in transfection.

III.18. DUAL LUCIFERASE REPORTER ASSAY

III.18.A. Cell Culture

The cells used for this study was human endothelial cells (EA.hy926 cells). The EA.hy926 endothelial cell line is a hybrid of HUVECs and A549 human lung carcinoma epithelial cells [257]. These cells were maintained in growth medium at 37°C and 5% CO_2 and were grown to confluence before being subcultured. Methods of subculturing of EA.hy 926 cells were similar to that of RAECs.

III.18.B. Transient transfection

For performing transient transfection, EA.hy 926 cells were plated at a density of 50000 cells per well in 4 well culture dishes. Transfection was carried out after 12-16 h post

plating by lipid mediated transfection using Lipofectamine 2000 cationic detergent. In one tube, 1 μg of plasmid vectors (pMCP-1 luc, pAP-1 Luc or pNFκB Luc) and 250 ng of pRL-TK were mixed in 125 μl of Opti-MEM and in another tube, 3 μl of Lipofectamine was mixed in 125μl of Opti-MEM. Both the plasmids and lipids were mixed together and incubated at room temperature for 45 minutes to allow formation of the lipid-DNA complex. During this period, the cells for transfection were washed with PBS to remove serum and incubated for about 45 minutes in Opti-MEM medium to condition the cells prior to transfection. Thereafter, the transfection mix was added to the cells and incubated for a period of 6 h to allow the cells take up the Lipid-DNA complex. Transfection mix was then replaced with 1ml of growth medium.

About 12 h after transfection, the EA.hy 926 cells were exposed to normal (5.5 mM) and high glucose concentration (25 mM) in the presence or absence of quercetin for a period of 6-24 h.

III.18.C. Reporter assay

Dual-luciferase reporter assay kit was used to quantify the luciferase activity of pMCP-1 Luc, pAP-1 Luc, pNFκB Luc and pRL-TK after transfection EA.hy 926 cells. As per manufacturer's instructions, the cells subjected to different treatments were lysed and the expression of luciferase reporter was quantitated as the luminence produced above background levels using a luminometer. The luciferase activity was normalized with pRL-TK. The luciferase activity in control group was taken as 100 % and the relative luciferase activity was calculated using the formula.

$$\frac{\textbf{Control luminescence units}}{\textbf{Treated luminescence units}} \textbf{ X 100}$$

III.19. STATISTICAL ANALYSIS

Each experiment was done at least 3 times and data were expressed as mean ± SD. Student's t-test was used for comparison between two groups and Oneway ANOVA (Bonferroni' Post Hoc analysis) was used for multiple groups. All analyses were done using SPSS software. Differences at $p<0.05$ were considered statistically significant.

IV. RESULTS

IV.1. ISOLATION, CULTURE AND CHARACTERIZATION OF RAT AORTIC ENDOTHELIAL CELLS (RAECs)

IV.1. Culture of RAECs

Cells started migrating from the edges of aortic rings 2 days after initiation of culture. The cells were polygonal with large oval shaped nuclei. After 7 days of culture initiation, there was marked proliferation of endothelial cells **(Figure 14 and 15)**. After 15 days, there were enough cells in the culture for passaging. Incubation of cells in medium containing D-valine ensured that only endothelial cells proliferated giving >95% pure culture. Cultures selected by medium containing D-valine retained normal endothelial cell morphology and endothelial markers. The procedure standardized for the isolation of RAECs is a less tedious involving no enzymatic digestion steps. The procedure was also found to be cost effective and the cells obtained were very healthy.

IV.2. Characterization of RAECs

The analysis of cell morphology, immunostaining with markers specific for endothelial cells and the uptake of DiI-Ac-LDL confirmed identity of the isolated cells as RAECs.

IV.2. A. Morphological analysis

Morphological analysis of the isolated RAEC cultures at confluence showed the typical cobblestone morphology which is a characteristic feature of endothelial cells **(Figure 16)**.

IV.2. B. Immunostaining

The RAEC cultures showed positive staining with antibody against factor VIII–relate antigen and vimentin, the markers for endothelial cells. Staining with antibody agains factor VIII–related antigen resulted as granules with typical perinuclear localizatio **(Figure 17)**, while staining for vimentin resulted in the whole of cytoplasm taking up th stain, characteristic of intermediate filaments **(Figure 18)**.

IV.2. C. Uptake of DiI-Acetylated LDL by RAECs

The RAECs incubated in medium containing DiI-Ac-LDL was able to take up DiI-Ac LDL within 4 h after exposure which is a characteristic feature of endothelial cells. The DiI fluorescence appeared to be granulated with a perinuclear distribution **(Figure 19)**.As RAECs take up DiI-Ac-LDL using the scavenger pathway for Ac-LDL metabolism, the result also suggests the cells to be functionally active.

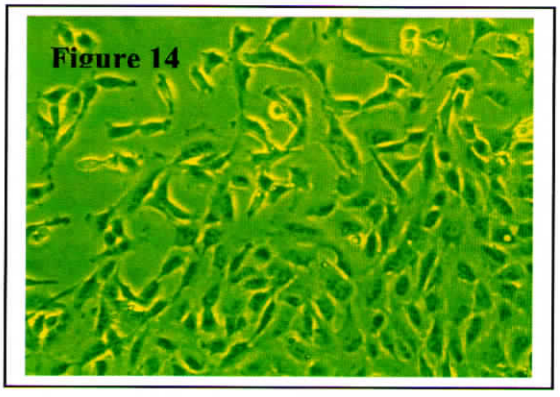


Figure 14: Photomicrograph of RAECs migrating from the aortic ring after 5 days of culture initiation. (100X)

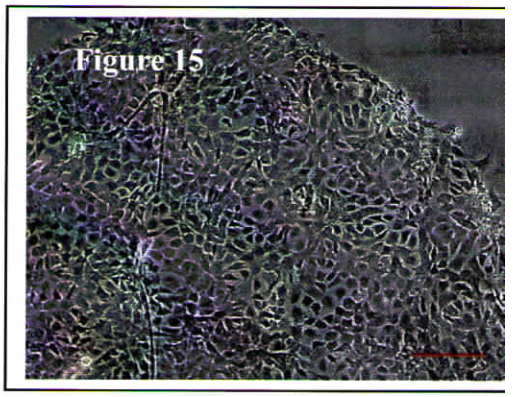


Figure 15: Photomicrograph of RAECs that migrated from the aortic ring after 10 days of cu initiation. The image was taken after removin aortic ring from the culture dish. Scale bar - 25 µ

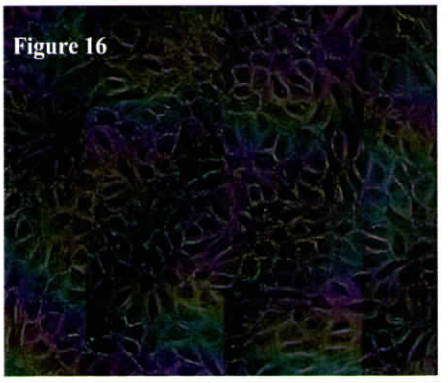


Figure 16: Photomicrograph of RAECs at confluence. (100X)

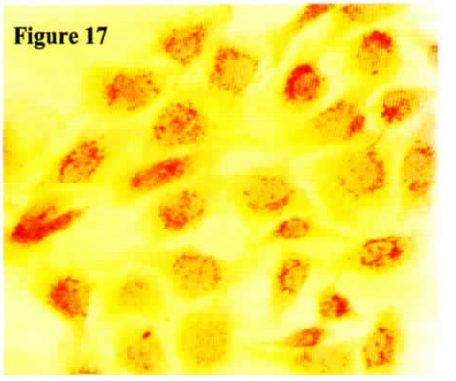


Figure 17: Photomicrograph of RAECs stained positive for factor VIII–related antigen. (200X)

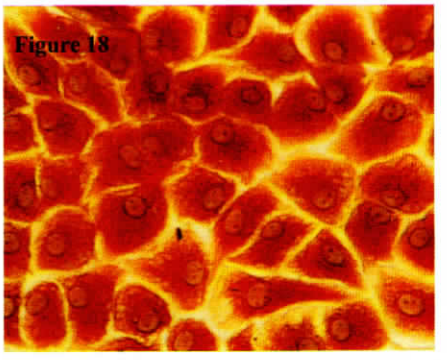


Figure 18: Photomicrograph of confluent monolayer of RAECs with cobblestone morphology stained positive for vimentin (200X).

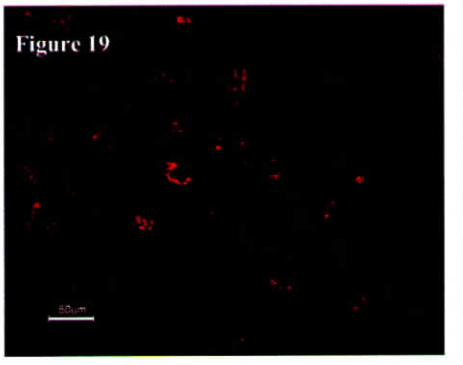


Figure 19: Fluorescence image of RAECs positive for DiI-Ac-LDL uptake. Scale bar- 50 µm.

IV.3. Growth kinetics of RAECs exposed to different glucose concentrations

The RAECs were analyzed for their growth pattern when cultured in medium containing various glucose concentrations by estimating their population doubling time (PDT). PDT of RAECs cultured in 25 mM (33.7 ± 1.97 h) and 35 mM (35.5 ±1.98 h) glucose medium was significantly longer ($p < 0.05$) when compared with that of 5.5 mM glucose medium (30.85± 1.76 h) **(Figure 20)**.

Figure 20: PDT of RAECs grown in different glucose concentrations

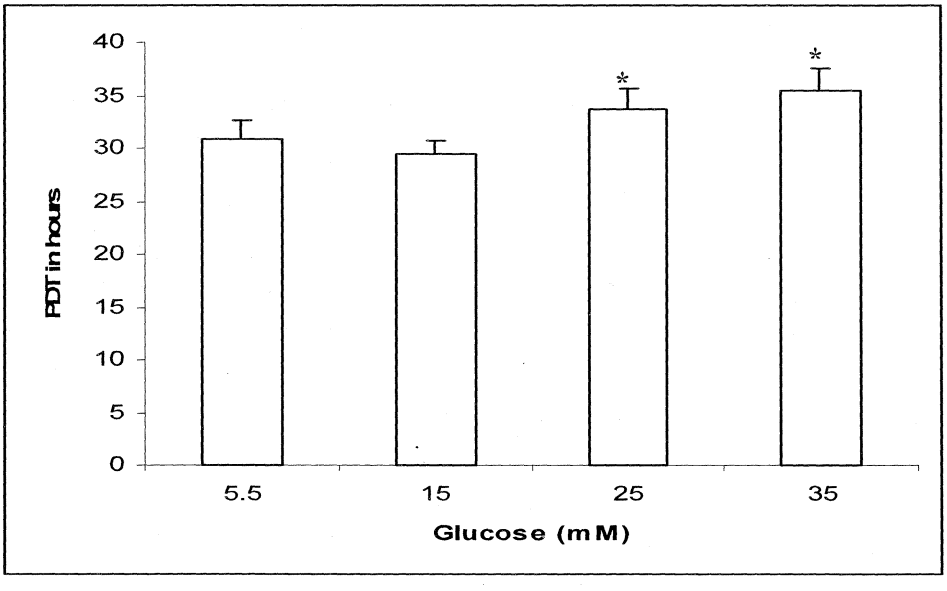


Figure 20: RAECs were grown in complete medium containing four different glucose concentrations and the PDT was estimated as described in the methods. All results are expressed as mean ± SD (n=4). *p< 0.05 vs control (5.5 mM). PDT- population doubling time.

IV.4. HG induces MCP-1 protein synthesis in a dose dependent manner

Before testing the effect of selected compounds in modulating the HG induced MCP-1 protein secretion by aortic endothelial cells, ELISA was done to analyze whether HG increased MCP-1 protein synthesis in a dose dependent manner. As compared with the control group (408± 78 pg/ml; 5.5 mM) there was a constant and significant increase in MCP-1 protein synthesis by RAECs exposed to higher glucose concentrations of 25 mM (1149± 122 pg/ml; $p< 0.05$) and 35mM (1989±134 pg/ml; $p<0.01$) at the end of 24 h exposure. This increase was in a dose dependent manner **(Figure 21)**.

Figure 21: Dose dependent effect of various glucose concentrations on MCP-1 protein secretion by RAECs

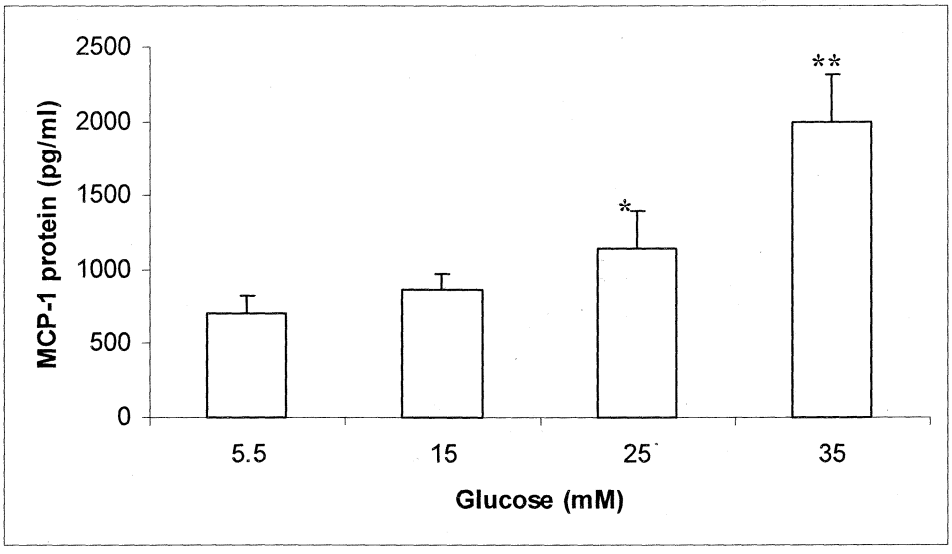


Figure 21: RAECs were cultured in medium with indicated glucose concentrations for 24 h and the MCP-1 protein levels in culture supernatants were determined by ELISA. All results are expressed as mean ± SD (n=3). *$p< 0.05$ and **$p<0.01$ vs control (5.5 mM).

IV.5. HG affects viability of RAECs

Higher glucose concentrations are reported to affect cell viability. Therefore, MTT assay was done in RAECs exposed to various glucose concentrations to rule out the possibility of glucose toxicity in the present experimental settings. The viability was not affected when RAECs were exposed to the four different glucose concentrations (5.5, 15, 25 and 35 mM) for a period of 24 and 48 h. When the cells were exposed to the above four glucose concentrations for 72 h, 35 mM glucose significantly ($p<0.05$ vs control) affected the viability of the cells **(Figure 22)**. Hence this concentration was not used for further studies. For all further studies 25 mM glucose concentration was used as HG treatment.

Figure 22: Effect of various glucose concentrations on RAEC viability

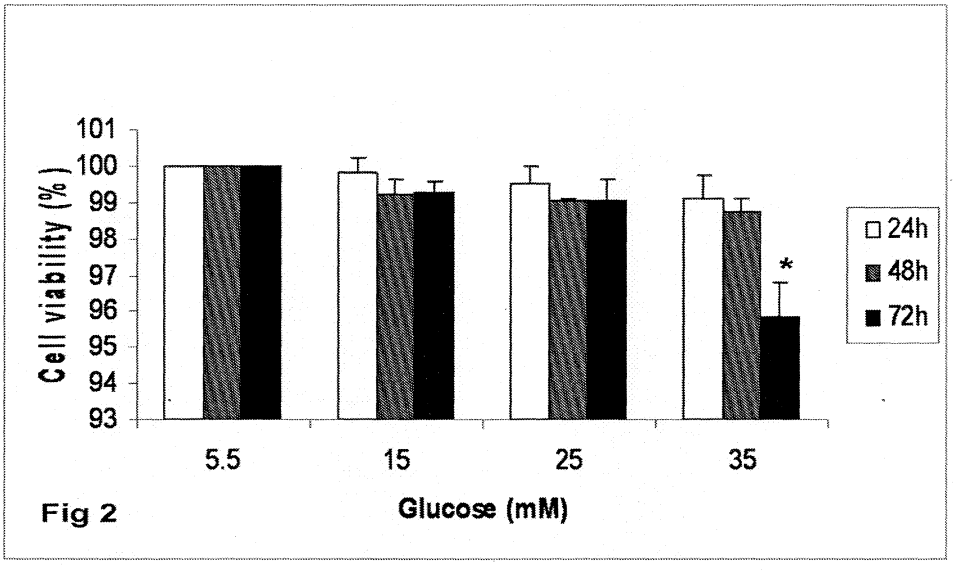


Figure 22: RAECs were cultured in medium with indicated glucose concentrations for 24, 48 and 72 h and the cell viability was studied using MTT assay. The OD in control group was taken as 100% of viability. All results are expressed as mean ± SD (n=3). *$p< 0.05$ vs control (5.5 mM).

IV.6. HG induces MCP-1 protein synthesis in a time dependent manner

After determining the effect of glucose concentrations on MCP-1 protein secretion, ELISA was done to analyze whether HG increased MCP-1 protein synthesis in time dependent manner. When RAECs were exposed to HG for 24 - 72 h, the MCP-1 protein secretion increased significantly ($p<0.05$ vs control) with respect to the duration of exposure to HG (1149±223 pg/ml at 24 h, 1864±323 pg/ml at 48 h and 2534±230 pg/ml at 72 h) **(Figure 23)**. D-mannitol (19.5 mM) used as osmotic control did not have any significant effect on MCP-1 protein synthesis by RAECs **(Figure 24)**.

Figure 23: Time dependent effect of HG on MCP-1 protein secretion by RAECs

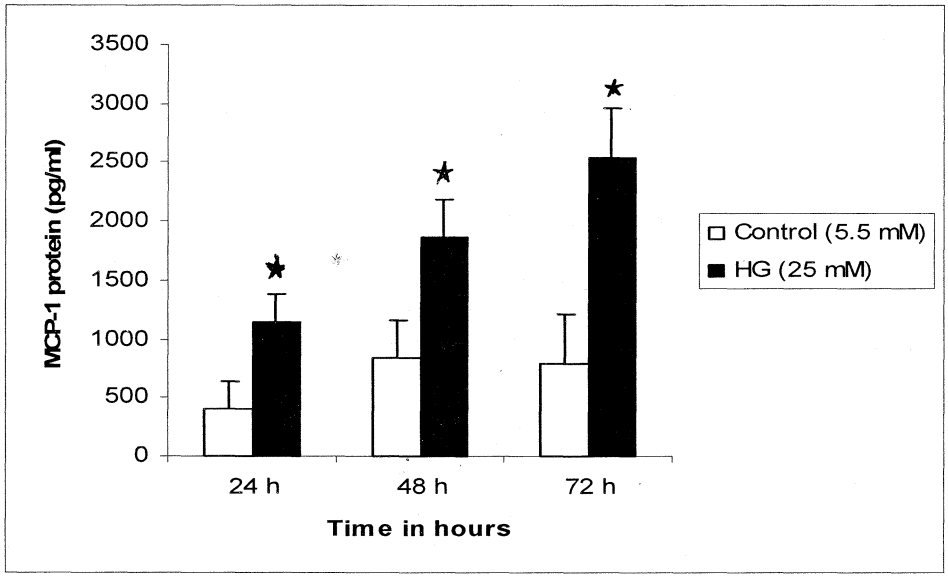


Figure 23: Time dependent effect of HG concentration on MCP-1 protein secretion by rat aortic endothelial cells (RAECs). RAECs were cultured in medium with HG (25 mM) concentrations for 24, 48 and 72 h and the MCP-1 protein levels in culture supernatants were determined by ELISA. All results are expressed as mean ± SD (n=4). *$p< 0.05$ vs control. HG- high glucose.

Figure 24: Effect of D-mannitol on MCP-1 protein secretion by RAECs

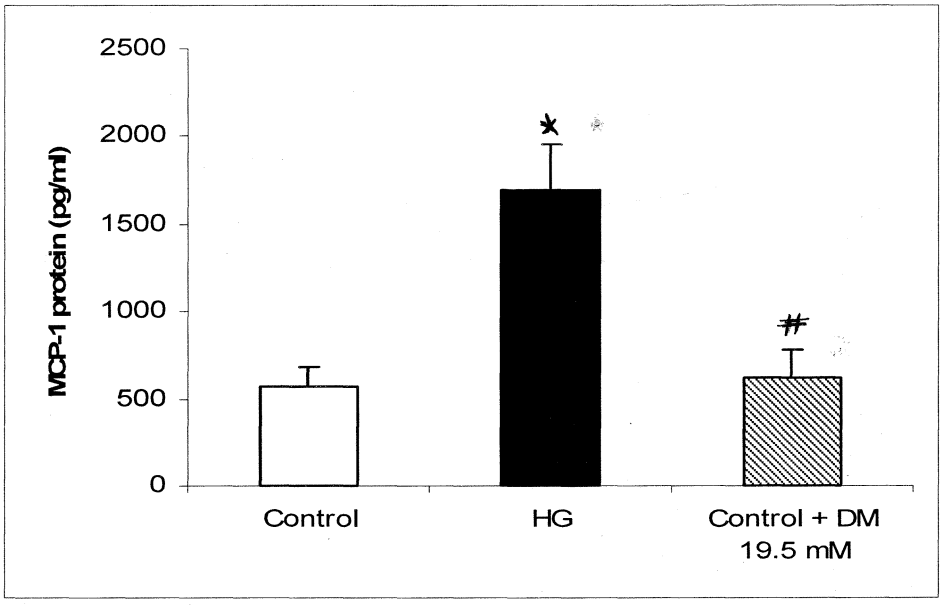


Figure 24: RAECs were cultured in control (5.5 mM) medium with D-mannitol (19.5mM) for 24 h and the MCP-1 protein levels in culture supernatants were determined by ELISA. All results are expressed as mean ± SD (n=3). # Not significant vs control; *$p<0.05$ vs control. HG- high glucose (25 mM); DM- D-mannitol.

IV.7. HG induces MCP-1 mRNA synthesis

To determine whether HG (25mM) induction of MCP-1 by RAECs was also complemented by prior increased MCP-1 mRNA synthesis, semi-quantitative RT-PCR was done. The results showed a significant increase in MCP-1 mRNA synthesis by RAECs exposed to HG as compared with that of control **(Figure 25.A and 25.B)**. Addition of 19.5 mM D-mannitol to control (5.5 mM) failed to increase MCP-1 mRNA levels when compared with that of control, ruling out osmolality as the reason for increased MCP-1 mRNA synthesis by RAECs upon HG stimulation.

Figure 25.A and 25.B: RT-PCR analysis of HG induced MCP-1 mRNA synthesis by RAECs

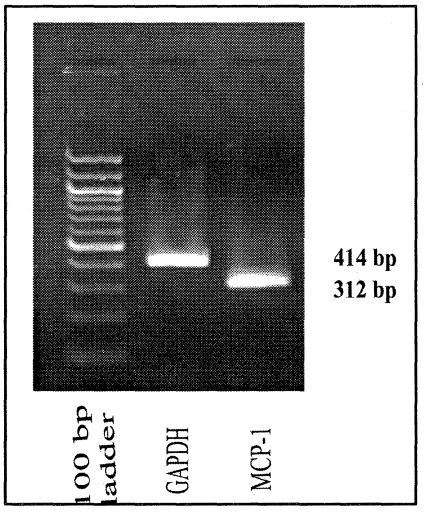


Figure 25.A. RT-PCR products for MCP-1 and GAPDH gene resolved on 1% agarose gel.

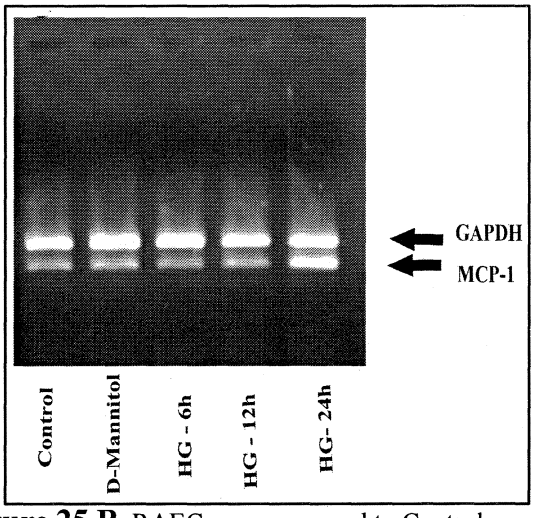


Figure 25.B. RAECs were exposed to Control (5.5mM), D-Mannitol (19.5mM) and HG (25mM) medium for upto 24 h and MCP-1 and GAPDH mRNA levels were determined by RT-PCR.. 5µl of each MCP-1 and GAPDH PCR products from each treatment group were resolved in 1% agarose gel and quantified using densitometry. HG exposure for upto 24 h was found to induce MCP-1 mRNA by nearly 1.5 fold over control.

IV.8. Losartan, genistein and sodium salicylate did not inhibit HG induced MCP-1 synthesis

The modulating effect of various selected molecules was determined using ELISA. RAECs subjected to HG treatments in the presence of losartan **(Figure 26)**, sodium salicylate **(Figure 27)** and genistein **(Figure 28)** at three different concentrations did not have any inhibitory effect on HG induced MCP-1 protein secretion.

Figure 26: Effect of losartan on HG induced MCP-1 protein secretion by RAECs

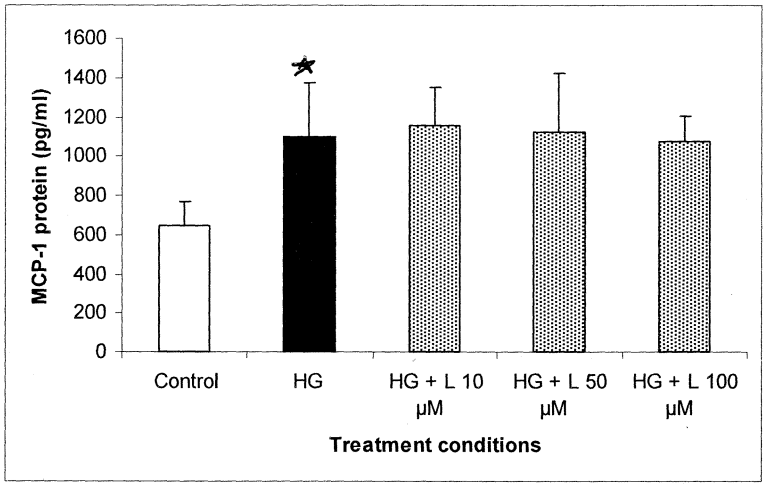


Figure 26: RAECs were cultured in medium with control (5.5 mM) and HG (25 mM) concentrations for 24 h in the absence or presence of indicated concentrations of losartan and the MCP-1 protein levels in culture supernatants were determined by ELISA. All results are expressed as mean ± SD (n=3). HG- high glucose, L- losartan.

Figure 27: Effect of sodium salicylate on HG induced MCP-1 protein secretion by RAECs

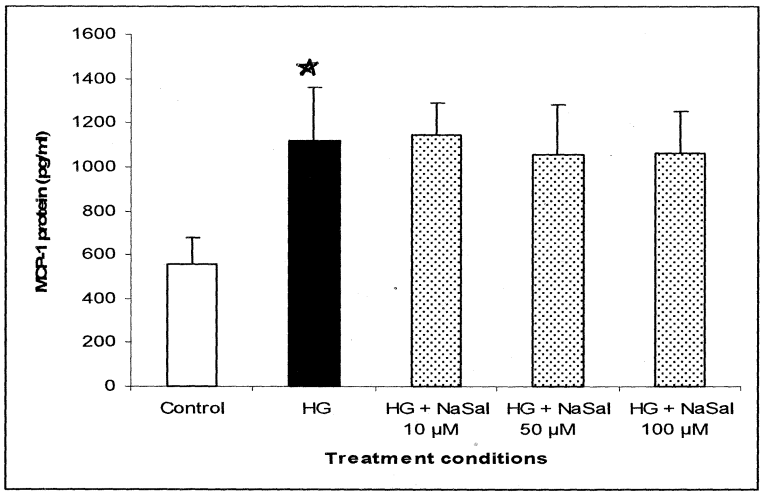


Figure 27: RAECs were cultured in medium with control (5.5 mM) and HG (25 mM) concentrations for 24 h in the absence or presence of indicated concentrations of sodium salicylate and the MCP-1 protein levels in culture supernatants were determined by ELISA. All results are expressed as mean ± SD (n=3). HG- high glucose, NaSal- sodium salicylate.

Figure 28: Effect of genistein on HG induced MCP-1 protein secretion by RAECs

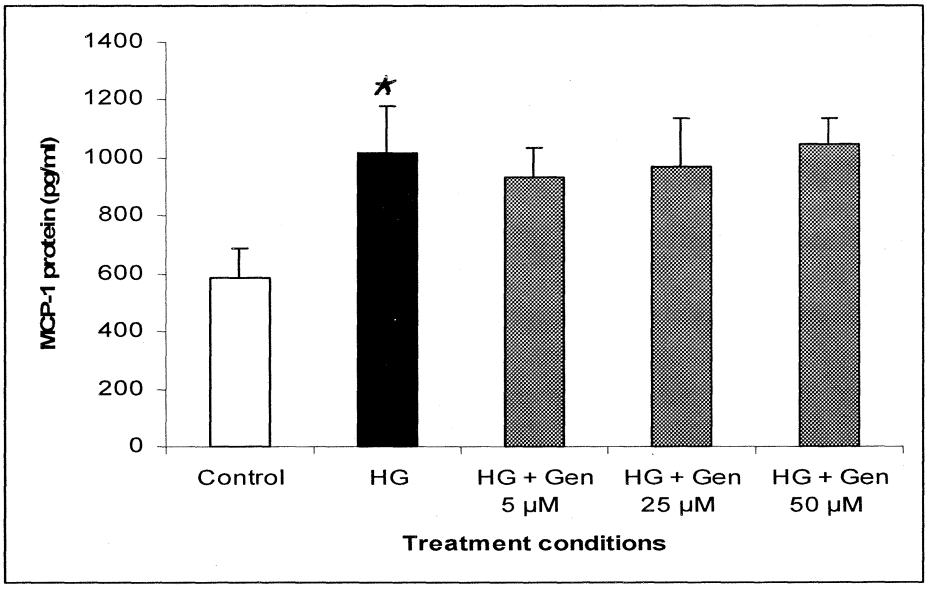


Figure 28: RAECs were cultured in medium with control (5.5 mM) and HG (25 mM) concentrations for 24 h in the absence or presence of indicated concentrations of genistein and the MCP-1 protein levels in culture supernatants were determined by ELISA. All results are expressed as mean ± SD (n=3). HG- high glucose, Gen- genistein.

IV.9. EFFECT OF CURCUMIN ON HG INDUCED MCP-1 SYNTHESIS IN RAECs

IV.9.A. Curcumin decreases HG induced MCP-1 protein and mRNA synthesis

Curcumin at 15 µM and 30 µM concentrations significantly attenuated HG induced MCP-1 protein synthesis in RAECs **(Figure 29)**. MCP-1 protein levels in culture supernatants of RAECs stimulated with HG in the presence of curcumin at 15 µM and 30 µM were 1169±147 pg/ml and 940±127 pg/ml respectively compared with MCP-1 protein levels in RAECs treated with HG only (1644±154 pg/ml).Thus, curcumin at 15 µM and 30 µM decreased HG induced MCP-1 secretion by about 40% ($p<0.05$) and 74% ($p<0.01$) respectively.

The mechanism of curcumin action at 30 μM concentration was further explored. Real-time RT-PCR analysis revealed that HG significantly increased MCP-1 mRNA synthesis by about 2 fold ($p<0.01$ vs control) and that curcumin attenuated the increased HG induced MCP-1 mRNA synthesis by about 80% ($p<0.05$ vs HG). The specific inhibitor of NFκB, Bay 11-7082 (5 μM) was added along with HG to check whether MCP-1 mRNA synthesis decreases due to NFκB inhibition. Similar to the effect of curcumin, Bay 11-7082 attenuated HG induced MCP-1 mRNA synthesis by about 53% ($p<0.05$ vs HG) **(Figure 30)**.

Figure 29: Effect of curcumin on HG induced MCP-1 protein secretion by RAECs

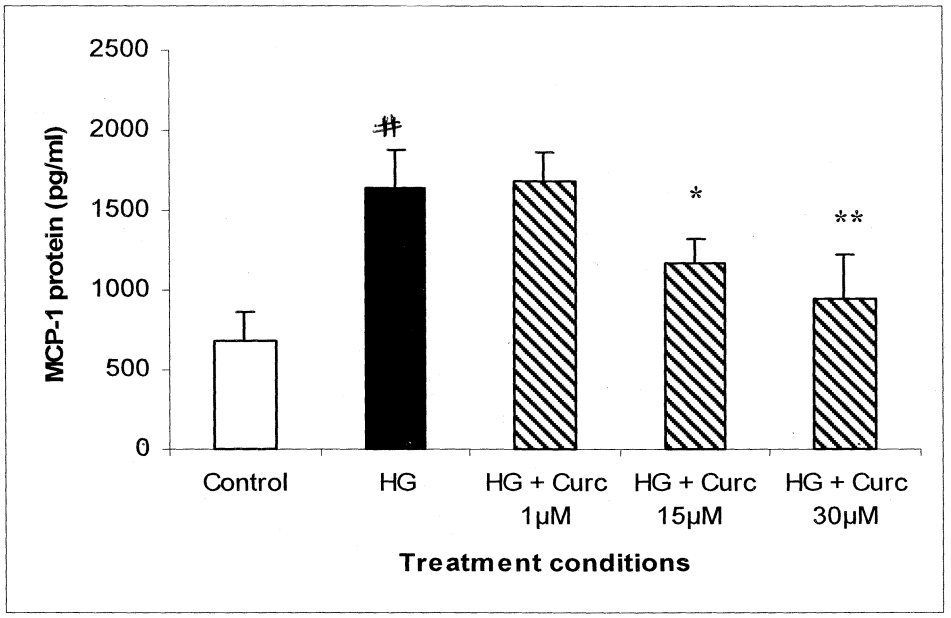


Figure 29: RAECs were cultured in medium with control (5.5 mM) and HG (25 mM) concentrations for 24 h in the absence or presence of indicated concentrations of curcumin and the MCP-1 protein levels in culture supernatants were determined by ELISA. All results are expressed as mean ± SD (n=4). *$p< 0.05$ and **$p<0.01$ vs HG. HG- high glucose, Curc- curcumin.

Figure 30: Effect of curcumin on HG induced MCP-1 mRNA synthesis by RAECs

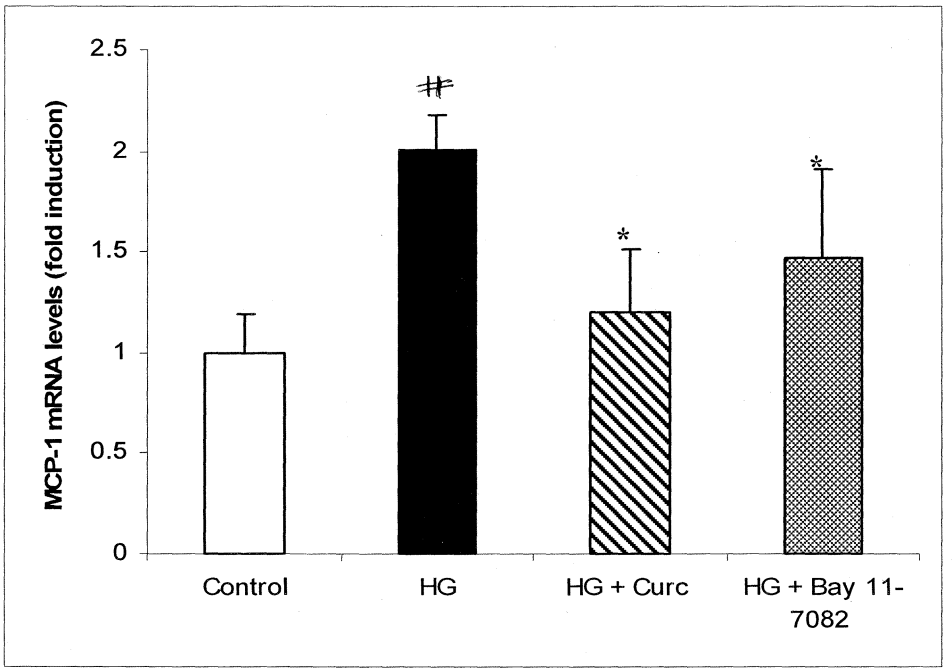


Figure 30: RAECs were cultured in medium with control (5.5 mM) and HG (25 mM) concentrations for 24 h in the absence or presence of curcumin (30 μM) or Bay 11-7082 (5 μM). MCP-1 and GAPDH mRNA levels were assessed by real time RT-PCR as described in methods. The relative amount of MCP-1 mRNA was normalized to GAPDH mRNA levels and the changes in mRNA levels were determined using the formula $2^{-\Delta\Delta Ct.}$ All results are expressed as mean ± SD (n=4). *p<0.05 vs HG. HG- high glucose, Curc-curcumin.

IV.9.B. Curcumin blocked HG induced NFκB DNA binding in RAECs

Increased DNA binding activity is part of NFκB activation. EMSA was done to analyze the DNA binding activity in HG stimulated RAECs. An increased band intensity of NFκB -DNA oligonucleotide complex was seen in EMSA suggesting increased NFκB binding to consensus DNA sequences upon HG stimulation. HG increased the band

intensity by about 2.4 fold as determined by densitometry. The addition of curcumin or Bay 11-7082 along with HG decreased the intensity of NFκB bands **(Figure 31)**. Curcumin reduced the HG induced NFκB binding activity to the baseline level as seen in control group. Bay 11-7082 almost completely abolished NFκB bands. Addition of excess cold unlabeled NFκB oligonucleotide completely abolished NFκB bands confirming the specificity of the NFκB binding activity.

Figure 31: Effect of curcumin on HG induced NFκB DNA binding activity

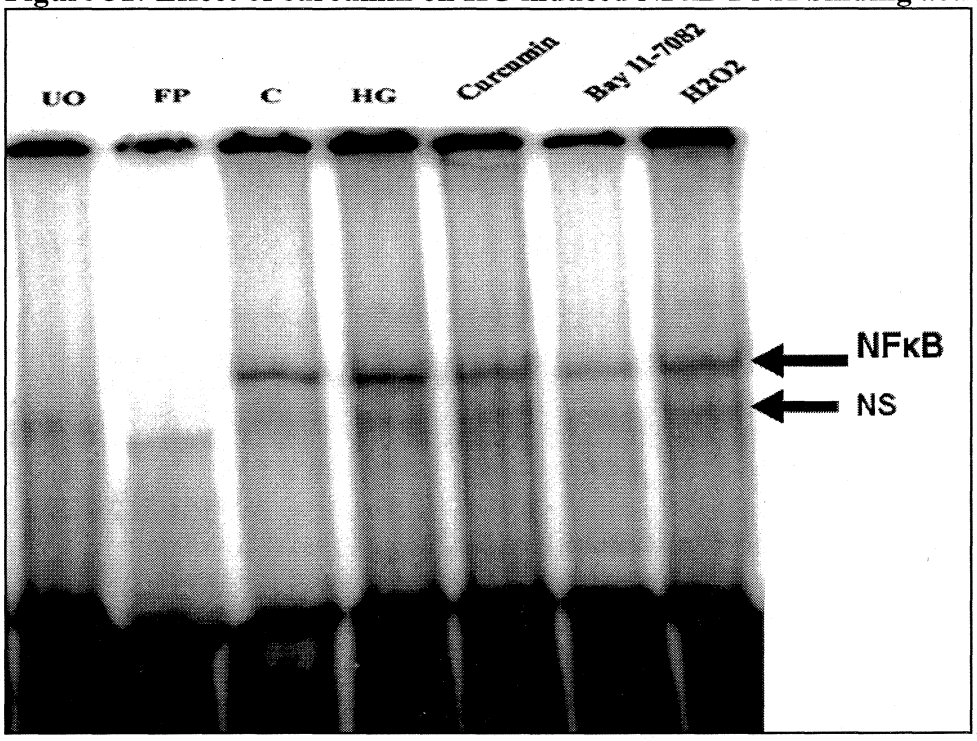


Figure 31: RAECs were cultured in medium with control glucose (5.5 mM) and HG (25 mM) concentrations for 24 h in the absence or presence of curcumin (30 μM) or Bay11-7082 (5 μM). H_2O_2 (10 μM) treated RAECs were used as positive control. Nuclear extracts were prepared and incubated with ^{32}P-labelled duplex NFκB oligonucleotide and the NFκB DNA binding activity was determined by EMSA. This is a representative of 3 separate experiments. UO- unlabelled oligo; FP- free probe; C- control; HG- high glucose; NS- non-specific band.

IV.9.C. Curcumin blocked HG induced p65 nuclear translocation in RAECs

The NFκB subunit, p65 was analyzed for their translocation into the nucleus upon HG stimulation. It was found that in control cells, the p65 proteins were predominantly localized in the cytoplasm itself and scantly present in the nucleus. Upon HG stimulation of RAECs, the p65 translocated from the cytoplasm to the nucleus. In the presence of curcumin, HG induced p65 nuclear translocation was almost completely inhibited as observed in confocal imaging **(Figure 32)**.

Figure 32: Effect of curcumin on HG induced p65 nuclear translocation in RAECs

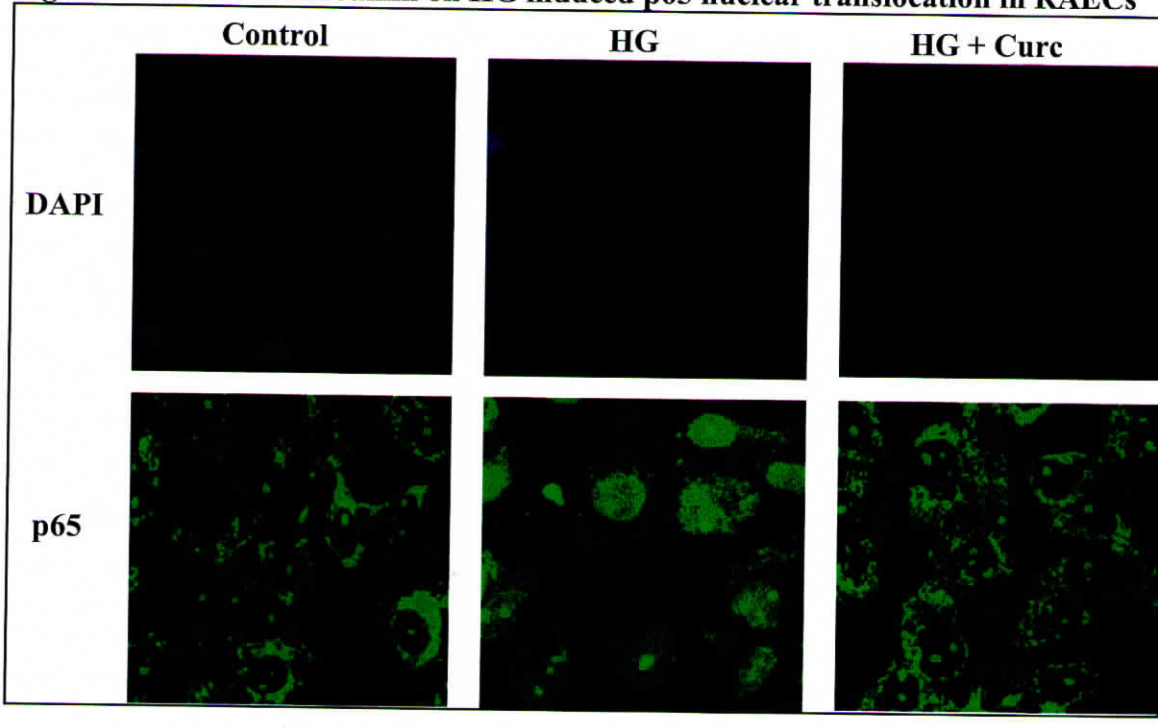


Figure 32. Curcumin blocked HG induced p65 nuclear translocation in rat aortic endothelial cells (RAECs). RAECs were stimulated with HG (25 mM) for 24 h in the presence or absence of curcumin (30 μM) and subsequently processed and analyzed for p65 nuclear translocation using confocal microscopy as described in the methods. DAPI was used to visualize the nucleus. This is representative of 3 separate experiments. HG- high glucose, Curc- curcumin, DAPI- 4-diamidino-2-phenylindole.

IV. 10. EFFECT OF QUERCETIN ON HG INDUCED MCP-1 SYNTHESIS IN RAECs

IV.10.A. Quercetin decreases HG induced MCP-1 protein synthesis

Similar to the effect of curcumin, quercetin also significantly attenuated HG induced MCP-1 mRNA and protein synthesis in RAECs. Quercetin attenuated HG induced MCP-1 protein synthesis in RAECs at concentrations of 50 and 100 μM **(Figure 33)**. MCP-1 protein levels in culture supernatants of RAECs stimulated with HG in the presence of quercetin at 50 μM and 100 μM were 975±124 pg/ml and 775±162 pg/ml respectively compared with MCP-1 protein levels in RAECs treated with HG only (1389±168 pg/ml).Thus, quercetin at 50 and 100 μM decreased HG induced MCP-1 secretion by about 42 % ($p<0.05$) and 79 % ($p<0.01$) respectively.

Figure 33: Effect of quercetin on HG induced MCP-1 protein secretion by RAECs

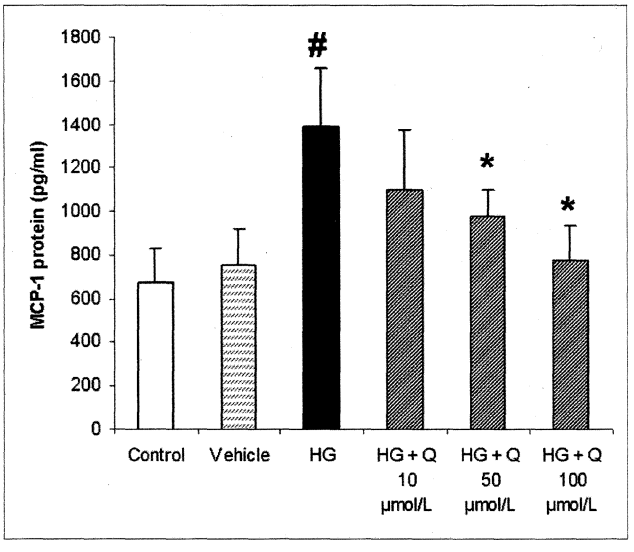


Figure 33: RAECs were cultured in medium with control (5.5 mM) and HG (25 mM) concentrations for 24 h in the absence or presence of indicated concentrations of quercetin and the MCP-1 protein levels in culture supernatants were determined by ELISA. All results are expressed as mean ± SD (n=4). *$p< 0.05$ vs HG. HG- high glucose, Q- quercetin.

IV.10.B. Quercetin and NAC differentially decreases HG induced MCP-1 mRNA synthesis and ROS generation in RAECs

As quercetin at 100 μM attenuated HG induced MCP-1 protein secretion in RAECs more efficiently, the mechanism of quercetin action at this concentration was further explored. Real-time RT-PCR analysis revealed that HG significantly increased MCP-1 mRNA synthesis by about 1.8 fold ($p<0.01$ vs control). Quercetin attenuated this HG induced increased MCP-1 mRNA synthesis by about 61% ($p<0.05$ vs HG). As ROS is suggested to be involved in HG mediated effects in endothelial cells, the specific inhibitor of ROS namely NAC (100 μM) was added along with HG to see whether MCP-1 mRNA synthesis goes down due to ROS inhibition. NAC attenuated HG induced MCP-1 mRNA synthesis by about 31 % ($p<0.05$ vs HG) but extent of inhibition was far less compared with that of quercetin (61%) **(Figure 34)**.

To qualitatively determine HG stimulated ROS generation in RAECs, the fluorescent DCFH-DA probe for intra-cellular ROS was used. The RAECs exposed to HG resulted in increased ROS generation as was evident from the intensely fluorescing cells whereas fluorescence was almost undetectable in cells exposed to normal glucose containing medium. NAC inhibited ROS generation to the basal level as in control. Similar to the effect of NAC, quercetin also inhibited ROS generation but the effect was not as strong as NAC. In quercetin treated group, fluorescing cells could be seen but the fluorescence was not as intense as in HG treated cells **(Figure 35)**.

Figure 34: Effect of quercetin and NAC on HG induced MCP-1 mRNA synthesis in RAECs

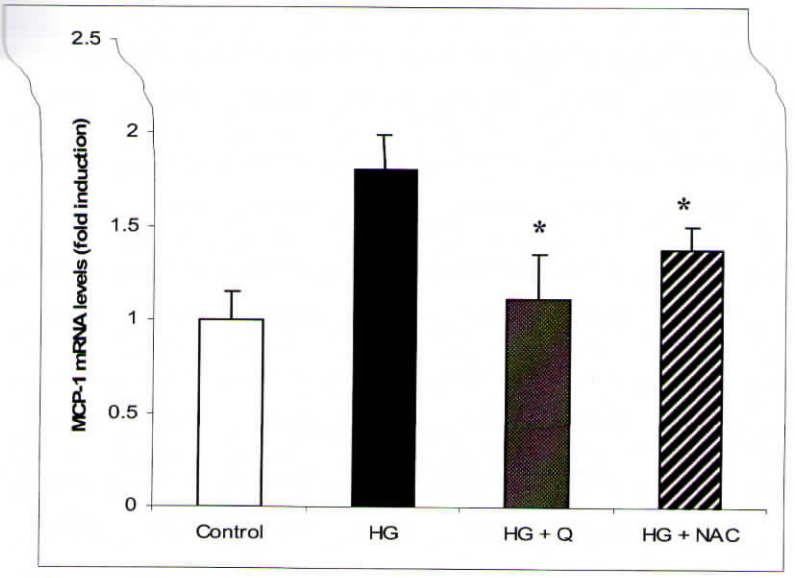


Figure 34: RAECs were cultured in medium with control (5.5 mM) and HG (25 mM) concentrations for 24 h in the absence or presence of quercetin (100 μM) or NAC (10 mM). MCP-1 and GAPDH mRNA levels were assessed by real time RT-PCR as described in methods. The relative amount of MCP-1 mRNA was normalized to GAPDH mRNA levels and the changes in mRNA levels were determined using the formula $2^{-\Delta\Delta Ct}$. All results are expressed as mean ± SD (n=4). *p<0.05 vs HG. HG- high glucose, Q- quercetin.

Figure 35: Effect of quercetin and NAC on HG induced ROS generation in RAECs (fluorescence imaging)

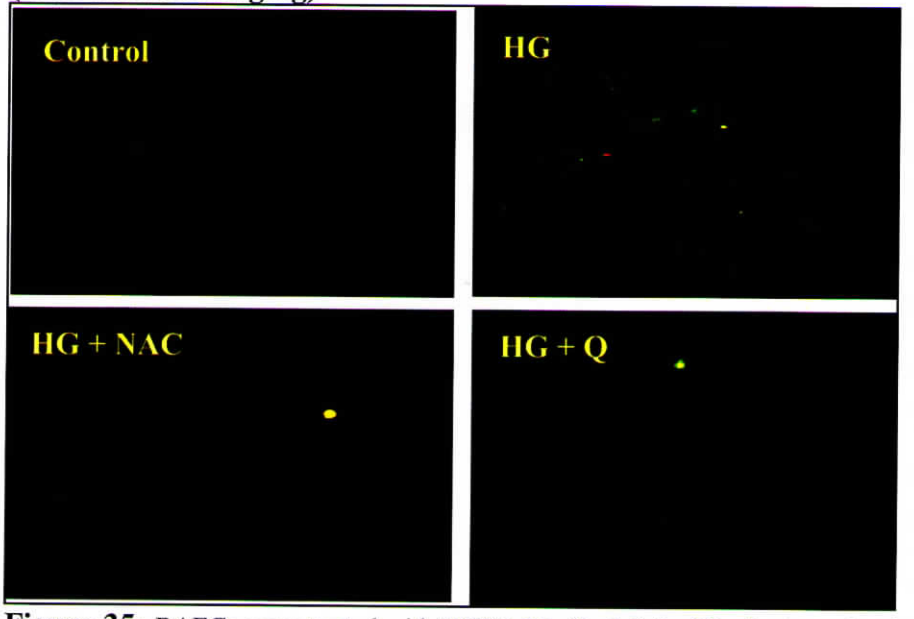


Figure 35: RAECs were treated with DCFH-DA (5 μM) for 30 minutes and stimulated with HG in the absence or presence of quercetin (100 μ M) or NAC (10 mM) for 2 h. Fluorescence emission was captured using fluorescence microscope at 485 nm excitation and 535 nm emission wavelength. HG- high glucose, Q- quercetin, NAC- N-acetyl cysteine.

IV.10.C. Quercetin prevents HG induced translocation of p65 from cytoplasm to the nucleus in RAECs

Activation of NFκB involves degradation of the NFκB regulatory protein, IκBα and the consequent translocation of NFκB subunits such as p65 into the nucleus where it mediates gene transcription. Western blot analysis of cytoplasmic proteins revealed that HG caused a decrease in the cytoplasmic levels of p65. In the presence of quercetin, the cytoplasmic p65 levles were seen to be similar to that of control. Similar to the effect of quercetin, NAC could also retain cytoplasmic p65 levels but not upto the basal levels as seen in the control **(Figure 36)**. Analysis of IκBα revealed that the levels of IκBα in the cytoplasm of control and HG treated groups were similar at the end of 24 h treatment. At the end of 24 h treatment, quercetin was found to significantly increase the levels of IκBα in the cytoplasm and these levels were higher than that seen in the control **(Figure 37)**. Confocal imaging of cells stained for p65 was done to evaluate nuclear translocation of p65 in treated cells. It was found that HG caused an increased nuclear translocation of p65 whereas in control group, the p65 was predominantly localized in the cytoplasm. In cells treated with quercetin, the p65 nuclear translocation was effectively blocked but in NAC treated cells there was no significant inhibition of HG induced nuclear translocation **(Figure 38)**.

Figure 36: Effect of quercetin and NAC on HG induced dynamics of cytoplasmic p65 in RAECs

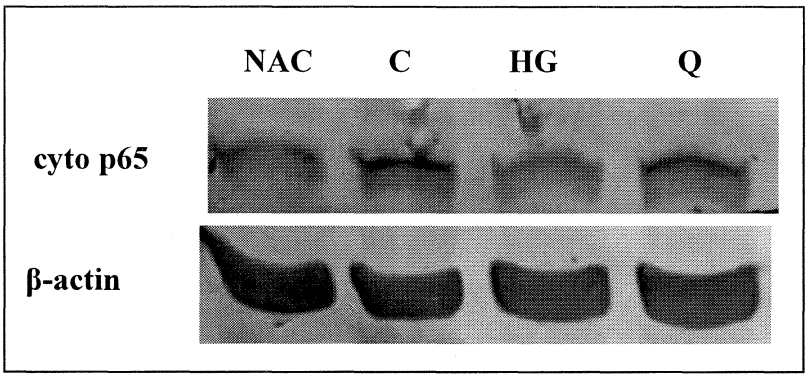


Figure 36: RAECs were cultured in control (5.5 mM) and HG (25 mM) medium for 24 h in the absence or presence of quercetin (100 μM) or NAC (10 mM). Cytoplasmic p65 levels were determined by western blotting as described in the methods. β-actin was used as loading control. This is a representative of 3 separate experiments. HG- high glucose, Q- quercetin, NAC- N-acetyl cysteine.

Figure 37: Effect of quercetin on HG induced cytoplasmic IκBα degradation in RAECs

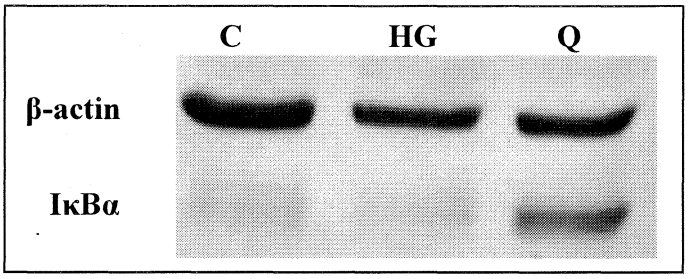


Figure 37: RAECs were cultured in control (5.5 mM) and HG (25 mM) medium for 24 h in the absence or presence of quercetin (100 μM). Cytoplasmic IκBα levels were determined by western blotting as described in the methods. β-actin was used as loading control. This is a representative of 3 separate experiments. HG - high glucose, Q - quercetin, NAC- N-acetyl cysteine.

Figure 38: Effect of quercetin and NAC on HG induced p65 nuclear translocation in RAECs (confocal imaging)

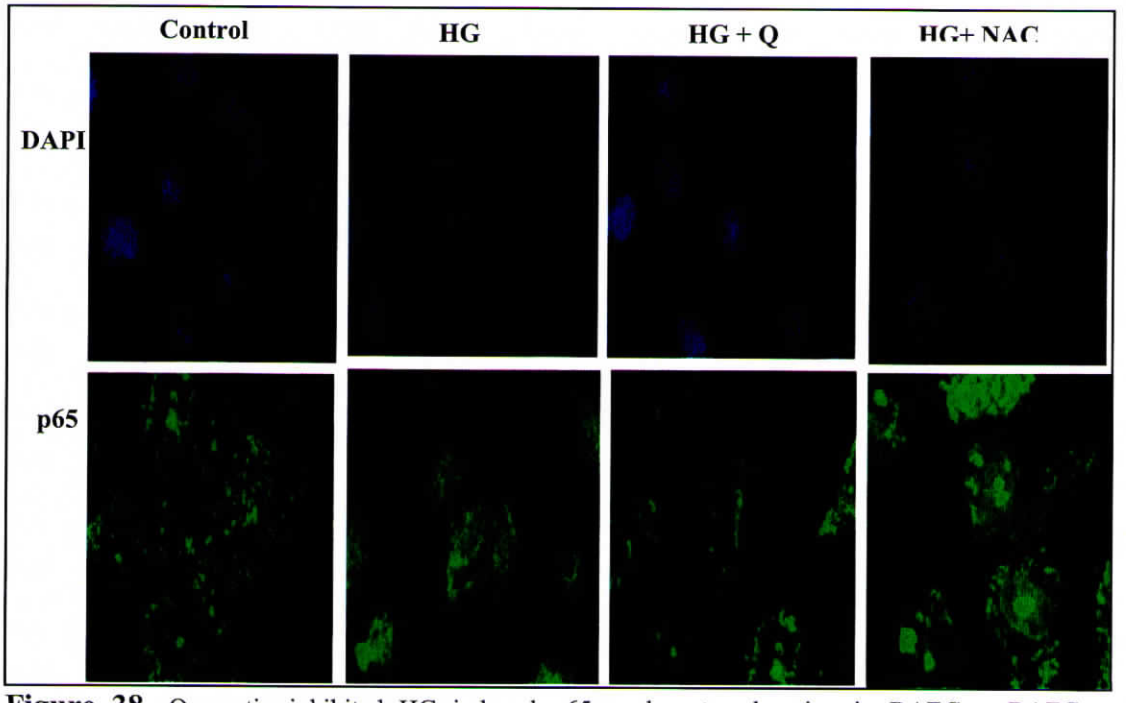


Figure 38. Quercetin inhibited HG induced p65 nuclear translocation in RAECs. RAECs were stimulated with HG (25 mM) for 12 h in the presence or absence of quercetin (100 μM) or NAC (10 mM) and subsequently processed and analyzed for p65 nuclear translocation using confocal microscopy as described in the methods. DAPI was used to visualize the nucleus. This is representative of 3 separate experiments. HG- high glucose, Q- quercetin, NAC- N-acetyl cysteine, DAPI- 4-diamidino-2-phenylindole

IV.10.D. Quercetin attenuates HG induced NFκB DNA binding activity in RAECs

NFκB activation involves increased DNA binding activity and EMSA was done to study this activity in HG stimulated RAECs. HG increased the band intensity of NFκB -DNA oligonucleotide complex by nearly 1.8 fold when quantified using densitometry suggesting increased NFκB binding to consensus DNA sequences upon HG stimulation. The addition of quercetin along with HG decreased the intensity of NFκB bands to the baseline level as seen in the control **(Figure 39)**. This suggests that quercetin attenuates

HG induced NFκB DNA binding activity. Addition of excess cold unlabeled oligonucleotide completely abolished NFκB bands confirming the specificity of the NFκB binding activity.

Figure 39: Effect of quercetin on HG induced NFκB DNA binding activity in RAECs

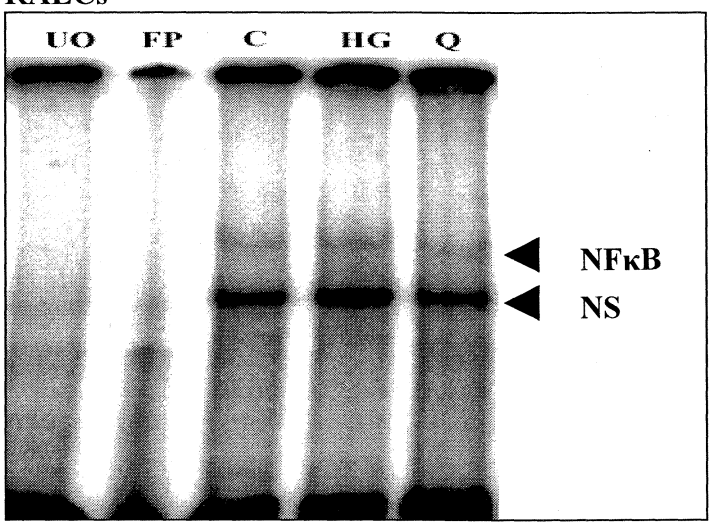


Figure 39: RAECs were cultured in medium with control glucose (5.5 mM) and HG (25 mM) medium for 24 h in the absence or presence of quercetin (100 μM). Nuclear extracts were prepared and incubated with ^{32}P-labelled duplex NFκB oligonucleotide and the NFκB DNA binding activity was determined by EMSA. This is a representative of 3 separate experiments. UO- unlabelled oligo, FP- free probe, C- control, HG- high glucose, NS- non-specific band.

The subunit composition of NFκB bands upon HG stimulation was determined by supershift analysis with antibodies against p65, p50 and c-rel. These proteins are reported to be present predominantly in the NFκB bands. Supershift analysis revealed that the NFκB bands were composed of p65 and p50 heterodimer, because in their presence, there was a clear shift in the NFκB bands. However, with c-rel there was no shift in the NFκB bands suggesting absence of c-rel in the DNA binding complex **(Figure 40)**.

Figure 40. Supershift analysis of NFκB bands

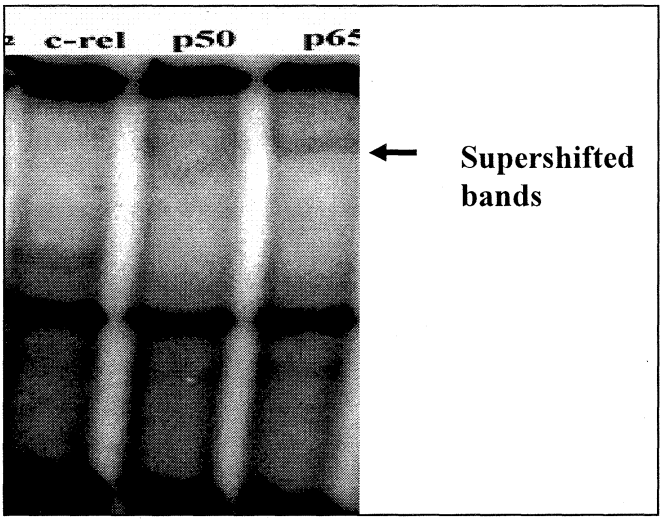


Figure 40: Supershift analysis was done as described in the methods using antibodies against p65, p50 and c-rel in nuclear extracts prepared from HG (25 mM) stimulated RAECs for 24 h. This is a representative of 3 separate experiments. Arrow denotes the supershifted bands.

IV.10.E. Quercetin attenuates HG induced AP-1 DNA binding activity in RAECs

Another transcription factor namely AP-1 is reported to be involved in MCP-1 gene regulation. EMSA was done to examine whether HG stimulates AP-1 DNA binding activity in RAECs. It was found that, similar to the effect of HG on NFκB, HG also increased AP-1 DNA binding activity. Three distinct AP-1 bands were obtained in EMSA whose intensity was different in control and HG. The upper and the lower bands were more intense whereas the middle band was less intense in HG treated group when compared with that of control. In the HG treated group, there was a 3.2 fold and 2 fold increase in the upper and the lower band intensity respectively when compared with that of control. However, there was a decrease in the intensity of the middle band by 1.8 fold

in HG treated group. Quercetin significantly attenuated the HG induced AP-1 DNA binding activity. Quercetin completely abolished the lower band and decreased the upper band intensity.The intensity of middle band was similar to that seen in the control **(Figure 41)**. Curcumin was also found to significantly inhibit AP-1 DNA binding in HG stimulated RAECs but not as effectively as quercetin.

Figure 41: Effect of quercetin on HG induced AP-1 DNA binding activity in RAECs

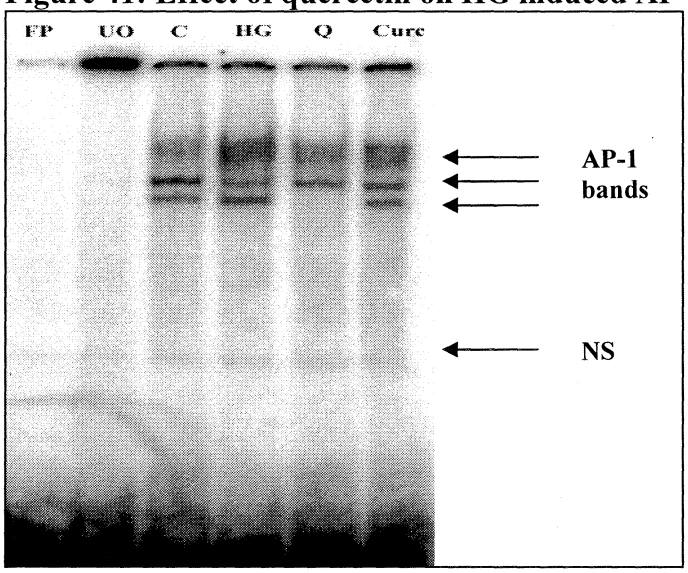


Figure 41: RAECs were cultured in medium with control glucose (5.5 mM) and HG (25 mM) medium for 24 h in the absence or presence of quercetin (100 μM). Nuclear extracts were prepared and incubated with ^{32}P-labelled duplex AP-1 oligonucleotide and the AP-1 DNA binding activity was determined by EMSA. This is a representative of 3 separate experiments. UO- unlabelled oligo, FP- free probe, C- control, HG- high glucose, Q-quercetin, Curc- curcumin, NS- non-specific band.

In order to find out the subunit composition of HG induced AP-1 bands, supershift analysis was done with antibodies against c-jun, c-fos and fra-1. It was found that the anti-c-jun antibody almost completely abrogated the upper and middle bands. In the presence of anti-fra-1 antibody, the middle band got nearly abrogated whereas the abrogation of the upper band was not as effective as seen with anti-c-jun antibody. Abrogation of the upper and middle band was also seen with anti-c-fos antibody but the effect was less efficient when compared with anti-c-jun and anti-fra-1 antibodies.

However, the lower band was abrogated to the same extent with all the above antibodies against three subunits of AP-1. These results suggest that the upper and the middle bands are predominantly composed of c-jun and fra-1 heterodimer. The results also suggest that the lower band may contain fra-1, c-jun or c-fos but they are not the predominant components of AP-1 in the lower band **(Figure 42)**.

Figure 42: Supershift analysis of AP-1 bands

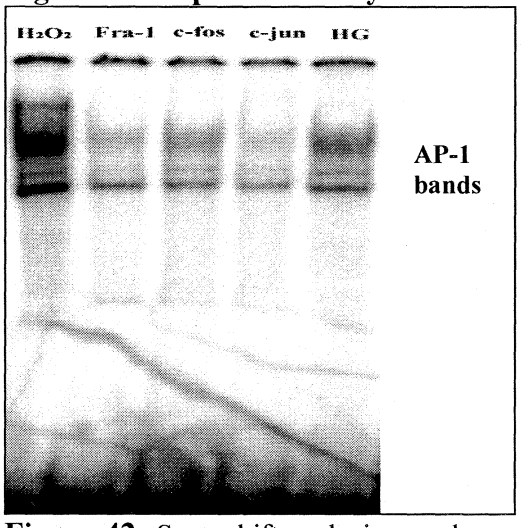


Figure 42: Supershift analysis was done as described in the methods using antibodies against c-jun, c-fos and fra-1 in nuclear extracts prepared from HG (25 mM) stimulated RAECs for 24 h. H_2O_2 (10 μM) treated RAECs were used as positive control. This is a representative of 3 separate experiments. HG- high glucose.

IV.10.F. Quercetin prevents HG induced c-jun nuclear localization in RAECs

As the antibody against c-jun subunit of AP-1 was found to abrogate HG induced AP-1 bands more efficiently, the effect of HG and quercetin on c-jun nuclear localization was explored using confocal imaging. It was found that HG increased the accumulation of c-jun in the nucleus and quercetin could block this accumulation. However NAC, the inhibitor of ROS, could not prevent the accumulation of c-jun in the nucleus of RAECs **(Figure 43)**.

Figure 43: Effect of quercetin on HG induced c-jun nuclear accumulation in RAECs (confocal imaging)

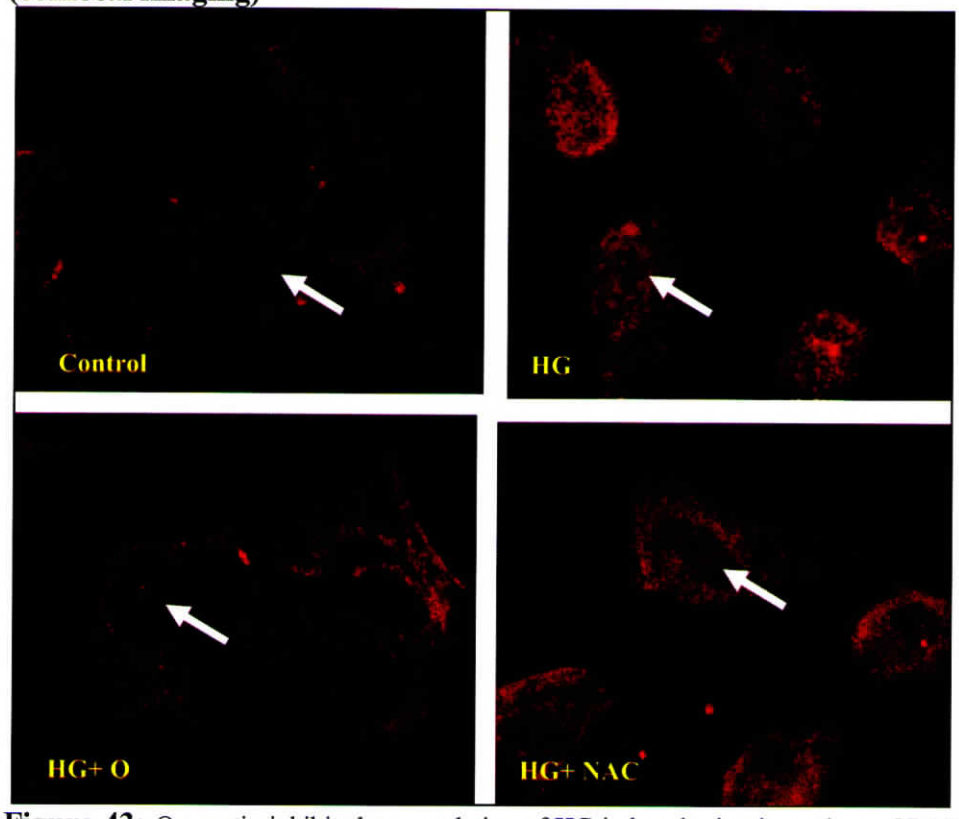
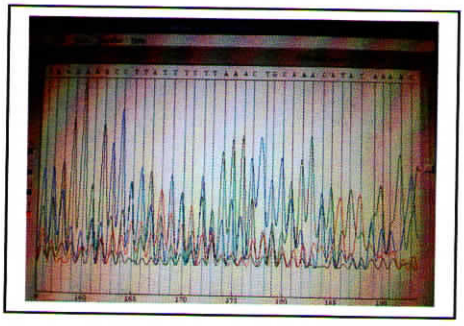


Figure 43: Quercetin inhibited accumulation of HG induced c-jun in nucleus of RAECs. RAECs were stimulated with HG (25 mM) for 12 h in the presence or absence of quercetin (100 μM) or NAC (10 mM)and subsequently processed and analyzed using confocal microscopy as described in the methods. Arrows indicate nucleus. This is representative of 3 separate experiments. HG- high glucose, Q- quercetin, NAC- N-acetyl cysteine.

Figure 45 A: Sequence analysis of plasmid DNA from AP-1 clone using GL2 primer

DNA sequencing result:



Chromatogram obtained after DNA sequencing

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TGACTAATTGAGATGCAGATCGCAGATCTCGAGGTGGAGTCAGGCAGGGTGGTTGGTGCCAAGGAGCAGCA
TCATCCTCGATGAGTGATGGAAAGTGGAAGTTTGAATCTGCTGAGTAAGTGCCGAGCCCTTGGATGTTTCC
AGGAAGTAGAAGGGAGAAACCGCGGATTGGAATTTGGTGTTTTTCCAGCCACTACTGGGAGCAAATGAAGC
TGCGCAGGGAGGGAGAAAAGAAGGAAGGGAGCAGTCGTACTAGACTGTGACCACAAGTGTCGGTACCTATC
GATAGAGAAATGTTCTGGCACCTGCACTTGCACTGGGGACAGCCTATTTTGCTAGTTTGTTTTGTTTCGTT
TTGTTTTGATGGAGAGCGTATGTTAGTACTATCGATTCACACAAAAAACCAACACACAGATGTAATGAACA
TAAAGATATTTTATTGCGGCCGCTCCAAGTACCTTCCCGTACCTTAATATTACTTACTTATCATGGTAGCT
TGGGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTT

The sequence highlighted in yellow is the AP-1 sequence. The AP-1 sequence in the pGL3 promoter is having 100% homology with that in the rat MCP-1 promoter when analyzed with BLAST (bl2seq) programme.

Score = 460 bits (239), Expect = 5e-126
Identities = 241/242 (99%), Gaps = 0/242 (0%)
Strand=Plus/Minus

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Query  246   GGTGGAGTCAGGCAGGGTGGTTGGTGCCAAGGAGCAGCATCATCCTCGATGAGTGATGGA  305
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Query  486   TC  487
              ||
Sbjct 3265  TC 3264
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IV.11. CLONING OF NFκB AND AP-1 ENHANCER SEQUENCES UPSTREAM OF MCP-1 GENE PROMOTER

The isolated pGL3 promoter vector became linearized after double digestion with Kpn1 and Xho1 **(Figure 44.A)**. The PCR for amplifying NFκB and AP-1 sequences upstream of MCP-1 gene promoter in the rat genomic DNA using specific primers resulted in the amplification of NFκB and AP-1 sequences **(Figure 44.B)**. Both, the double digested PCR products (inserts) and the pGL3 promoter vector were purified and successfully ligated. The successful ligation was evident from the ampicillin resistant colonies that came up after transformation of ligation mix. The results of the sequence analysis of plasmid DNA isolated from most of the positive clones in each of the NFκB and AP-1 group revealed 100 % homology with those present upstream of the rat MCP-1 gene promoter **(Figure 45.A and 45.B)**. Glycerol stock of a few NFκB and AP-1colonies having 100 % homology was made. Plasmid DNA isolated from one of glycerol stock in each group was used for the entire enhancer activation studies.

Figure 44 A and 44 B: Restriction digestion of pGL3 promoter vector and PCR amplification of NFκB and AP-1 enhancer sequence.

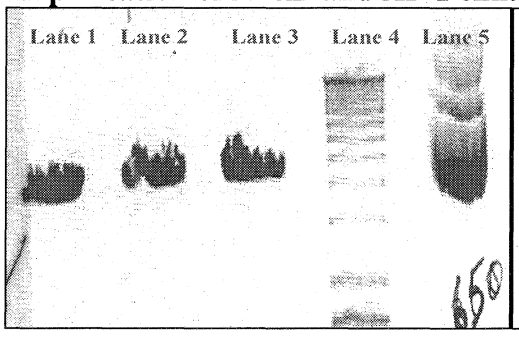


Figure 44 A: Double digested pGL3 promoter vector. Lane 1- Kpn1 digest, lane 2- Xho1 digest, Lane 3- Kpn1+Xho1 digest, Lane 4- 1Kb ladder and Lane 5- undigested pGL3 promoter vector.

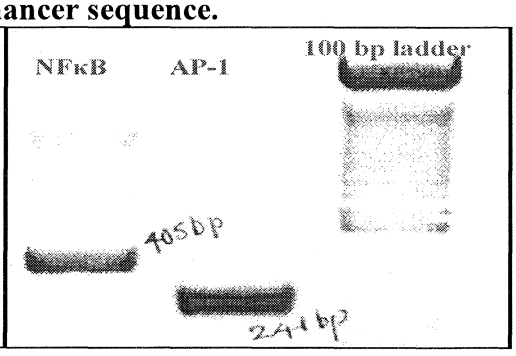
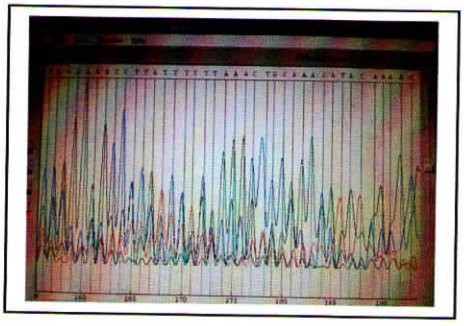


Figure 44 B: PCR products for NFκB (405 bp) and AP-1 (241 bp) enhancer sequences amplified from rat genomic DNA and resolved on 1% agarose gel.

Figure 45 A: Sequence analysis of plasmid DNA from AP-1 clone using GL2 primer

DNA sequencing result:



Chromatogram obtained after DNA sequencing

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TGACTAATTGAGATGCAGATCGCAGATCTCGAGGTGGAGTCAGGCAGGGTGGTTGGTGCCAAGGAGCAGCA
TCATCCTCGATGAGTGATGGAAAGTGGAAGTTTGAATCTGCTGAGTAAGTGCCGAGCCCTTGGATGTTTCC
AGGAAGTAGAAGGGAGAAACCGCGGATTGGAATTTGGTGTTTTTCCAGCCACTACTGGGAGCAAATGAAGC
TGCGCAGGGAGGGAGAAAAGAAGGAAGGGAGCAGTCGTACTAGACTGTGACCACAAGTGTCGGTACCTATC
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TAAAGATATTTTATTGCGGCCGCTCCAAGTACCTTCCCGTACCTTAATATTACTTACTTATCATGGTAGCT
TGGGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTT

The sequence highlighted in yellow is the AP-1 sequence. The AP-1 sequence in the pGL3 promoter is having 100% homology with that in the rat MCP-1 promoter when analyzed with BLAST (bl2seq) programme.

Score = 460 bits (239), Expect = 5e-126
Identities = 241/242 (99%), Gaps = 0/242 (0%)
Strand=Plus/Minus

```
Query  246   GGTGGAGTCAGGCAGGGTGGTTGGTGCCAAGGAGCAGCATCATCCTCGATGAGTGATGGA  305
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              ||
Sbjct 3265  TC 3264
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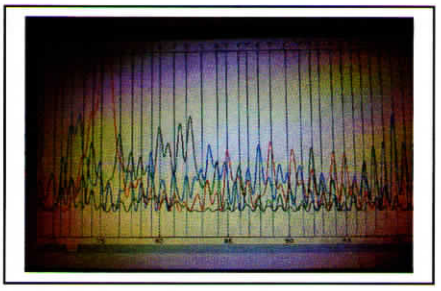
Figure 45 B: Sequence analysis of plasmid DNA from NFκB clone using RV3 primer

DNA sequencing result:



Chromatogram obtained after DNA sequencing

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TTCCTGGAACAATTGCTTTTACAGATGCACATATCGAGGTGGACATCACTTACGCTGAGTACTTCGAAATGTCCGTTCGGTTGGCAGAA
GCTATGAAACGATATGGGCTGAATACAAATCACAGAATCGTCGTATGCAGTGAAAACTCTTCTTCAATTCTTTATGCCGTGTGGGCGCG
TATTTATCGGAGTGCAGTTGCGCCGCGACGACATTTATAATGACGTGAATGCTCAACAGTATGGGCATTTCGCAGCTACGTGGTGTCGT
TTCAAAGGGTGCCAAAATTGGACGTGCAAAAAGCTCATCATCAAAATATATCATGATTAAACGATAACAGATCAGTCAGTTAACGTTGT
CAATCAATACTCGTATGATCGATTGCGAGCTCATGGCAGACATGACGTACTGACTCTGGAGTCTCACATGATTGCGCTAAAGATGACTC

The sequences highlighted in yellow is the NFκB sequence (site A and site B). The NFκB sequence in the pGL3 promoter is having 100% homology with that in the rat MCP-1 promoter when analyzed with BLAST (bl2seq) programme.

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Score =  771 bits (401),  Expect = 0.0
Identities = 405/407 (99%), Gaps = 0/407 (0%)
Strand=Plus/Plus


Query  100   TTTCCAAGTCAGAGCTCAGACTATGCCTTTGTTGAGCTATTCCAGATTCTCAGGCCCTTG  159
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1136  TTTCCAAGTCAGAGCTCAGACTATGCCTTTGTTGAGCTATTCCAGATTCTCAGGCCCTTG  1195

Query  160   TGAGAGCTGCTTGGCTGTAAGCCCAGCATCTGGAGCTCATATTCCAGCTAAATATCTCTC  219
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1196  TGAGAGCTGCTTGGCTGTAAGCCCAGCATCTGGAGCTCATATTCCAGCTAAATATCTCTC  1255

Query  220   CTGAAGGGTCTGGGAACTTCCAATGCTGCCTCAGAATGGGAATTTCCACACTCTTATCCT  279
             |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  1256  CTGAAGGGTCTGGGAACTTCCAATACTGCCTCAGAATGGGAATTTCCACACTCTTATCCT  1315

Query  280   ACTCTGCCTCTGACCTACCAGCTGGGAAGAGCATCCTTTGTTGACAGAGTAAAGTGAGTG  339
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1316  ACTCTGCCTCTGACCTACCAGCTGGGAAGAGCATCCTTTGTTGACAGAGTAAAGTGAGTG  1375

Query  340   GGAGAGAGACAATTATTTTCCTTTCTTTCGTTTATGATTCATACTGTGTTGCATCGTATG  399
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1376  GGAGAGAGACAATTATTTTCCTTTCTTTCGTTTATGATTCATACTGTGTTGCATCGTATG  1435
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IV.12. EFFECT OF QUERCETIN ON HG INDUCED MCP-1 PROMOTER AS WELL AS NFκB AND AP-1 ENHANCER ACTIVITIES

The entire transient transfection and luciferase reporter assay were done on EA.hy 926 cells as an alternative for RAECs because the transfection efficiency was very low (< 5%) in RAECs. These cells well known to respond to HG by increasing MCP-1 production and had good transfection efficiency (>20%). Hence these cells were suitable for present transfection studies.

IV.12.A. QUERCETIN ATTENUATES HG INDUCED MCP-1 PROMOTER ACTIVITY

To explore the transcriptional activity of HG induced MCP-1 gene in EA.hy 926 cells, the pMCP-1-Luc plasmid containing the 2.5 kb 5' flanking region of the mouse MCP-1 gene spanning -2466 to +67 was used for transfection studies. These sequences contain the two putative NFκB sites and one AP-1 site which were 100% identical with those in the rat MCP-1 gene promoter.

Consistent with the increased MCP-1 mRNA levels, in cells transfected with pMCP-1-luc plasmids, the MCP-1 gene promoter activity significantly increased by 73 % (HG: 173 ± 34% vs control: 100%). Quercetin significantly reduced HG induced MCP-1 promoter activity by about 75 % (Q, 129±39% vs HG, 173 ± 34%) **(Figure 46)**.

IV.12.B. QUERCETIN ATTENUATES HG INDUCED NFκB AND AP-1 ENHANCER ACTIVITIES

To explore the HG mediated activation of NFκB and AP-1 enhancers in EA.hy 926 cells, transfection studies were done with pNFκB-Luc and pAP-1-Luc plasmids respectively. These promoter constructs gave the activation of enhancers only and did not demonstrate the direct effect of NFκB and AP-1 enhancers on MCP-1 promoter activity.

HG induction of EA.hy 926 cells transfected with p-NFκB-Luc caused increased activation of NFκB by about 54% (HG: 154 ±39% vs control: 100%). In the presence of quercetin, the HG induction of NFκB was reduced by about 77% (Q: 119±31% vs HG: 154± 39%) **(Figure 47)**. Similarly, in cells exposed to HG after transfection with pAP-1-Luc, AP-1 activity increased by about 179% (HG: 279 ±71% vs control: 100%). Quercetin attenuated this HG induced AP-1 activation by about 57% (Q: 159 ±31% vs HG: 279 ±71%) **(Figure 48)**.

Figure 46: Effect of quercetin on HG induced MCP-1 promoter activity

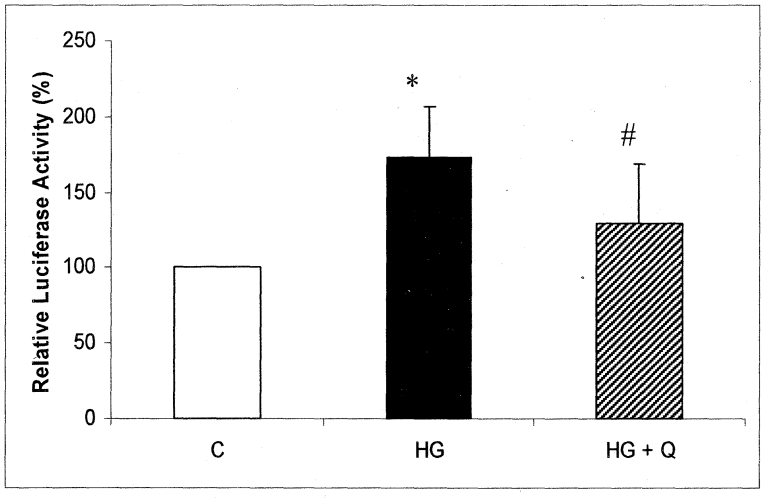

Figure 46: EA.hy 926 cells were transiently transfected with pMCP-1 luc as described in the methods. The cells were then stimulated with HG (25mM) in the presence or absence of quercetin (100 μM) for 12 h. The cells were subsequently lysed and the luciferase activity was measured as described in the methods. The luciferase activity of control was taken as 100 %. All results are expressed as mean ± SD (n=3). *p< 0.05 vs control (5.5 mM), #p< 0.05 vs HG (25 mM). HG- high glucose, Q- querectin.

Figure 47: Effect of quercetin on HG induced AP-1 enhancer activity

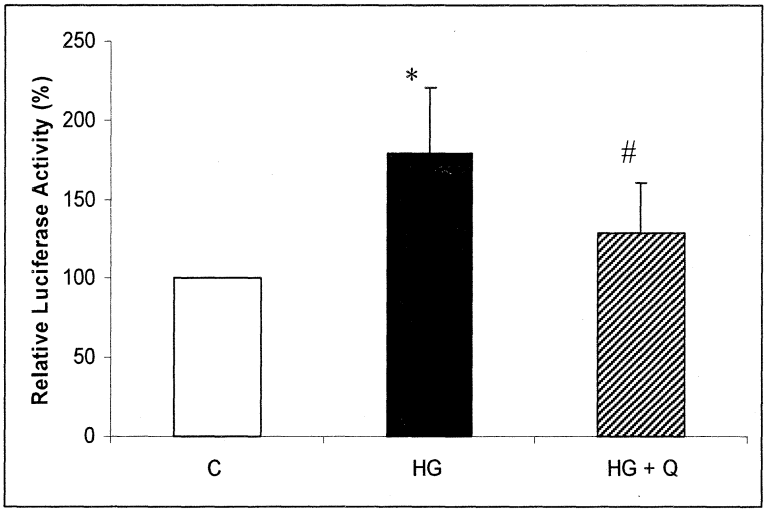

Figure 47: EA.hy 926 cells were transiently transfected with pAP-1 luc as described in the methods. The cells were then stimulated with HG (25mM) in the presence or absence of quercetin (100 μM) for 12 h. The cells were subsequently lysed and the luciferase activity was measured as described in the methods. The luciferase activity of control was taken as 100 %. All results are expressed as mean ± SD (n=3). *p< 0.05 vs control (5.5 mM), #p< 0.05 vs HG (25 mM). HG- high glucose, Q- querectin.

Figure 48: Effect of quercetin on HG induced NFκB enhancer activity

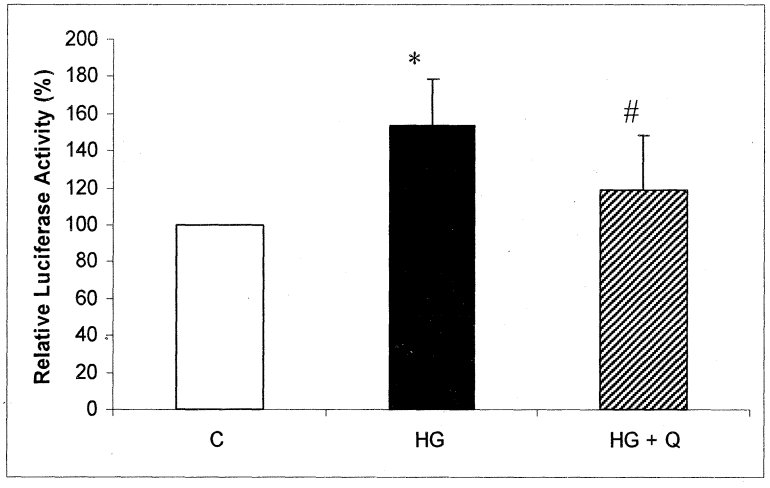

Figure 48: EA.hy 926 cells were transiently transfected with pNFκB-1 luc as described in the methods. The cells were then stimulated with HG (25mM) in the presence or absence of quercetin (100 μM) for 12 h. The cells were subsequently lysed and the luciferase activity was measured as described in the methods. The luciferase activity of control was taken as 100 %. All results are expressed as mean ± SD (n=3). *p< 0.05 vs control (5.5 mM), #p< 0.05 vs HG (25 mM). HG- high glucose, Q- querectin.

IV. DISCUSSION

Endothelial dysfunction mediated by hyperglycemia is a key feature of type 2 diabetes and is thought to be the major cause of vascular complications associated with diabetes [2, 3]. One of the primary mechanism by which hyperglycemia induced endothelial dysfunction may cause accelerated rate of atherosclerosis in diabetes is by the increased production of monocyte chemoattractant protein-1 (MCP-1). During the course of atherogenesis, synthesis of MCP-1 by endothelial cells is essential for the adhesion and migration of monocytes into the arterial intima. MCP-1 is already implicated both in the initiation and progression of atherosclerosis [7]. Therefore, the modulation of endothelial function by hyperglycemia leading to MCP-1 synthesis in patients with diabetes demands scrutiny.

Given the significant role of MCP-1 in the pathogenesis of atherosclerosis, glucose induced increased MCP-1 synthesis in vascular endothelial cells has been implicated in the pathogenesis of accelerated development of vascular lesions in diabetes mellitus [147]. High concentration of glucose (HG) is known to induce MCP-1 synthesis in aortic endothelial cells through the nuclear factor κB (NFκB). HG is also known to activate another important transcription factor namely activator protein-1 (AP-1) which has got putative binding sites upstream of MCP-1 gene promoter.

The present study was carried out to examine the regulation of MCP-1 gene in aortic endothelial cells upon HG stimulation. The study primarily focused on the regulation of MCP-1 gene by NFκB and AP-1 in HG stimulated rat aortic endothelial cells (RAECs). The study also examined the modulating effect of a set of selected pharmacological inhibitors on HG induced MCP-1 expression and delineated their possible mechanism of action.

V.1. ISOLATION AND CHARACTERIZATION OF RAT AORTIC ENDOTHELIAL CELLS (RAECs)

The endothelial cells including the RAECs are known to exhibit cobblestone morphology at confluence [245]. They express Factor VIII-related antigen [258] and have the ability to take up DiI-Ac-LDL within 2 to 4 h using specific scavenging receptors [246]. These features are absent in fibroblasts and vascular smooth muscle cells (VSMCs), the other cell types present in the aorta. The cells isolated from rat aorta had cobblestone morphology at confluence and not the 'hill and valley' pattern of VSMCs ruling out the possibility of the isolated cells being VSMCs. The isolated cells from rat aorta were also positive for the presence of Factor VIII-related antigen and uptake of DiI-Ac-LDL confirming the cells to be endothelial cells. One modification made by me in the method described by McGuire and Orkin for the culture of RAECs was the introduction of D-valine instead of L-valine in the culture medium after 1st passage for about 2 weeks. D-valine can be metabolized by the endothelial cells but not by the contaminating fibroblasts and VSMCs [259, 260]. The fact that the isolated cells could grow efficiently in D-valine containing medium confirmed the cells to be endothelial cells. This modification could also ensure the increased purity of the RAEC culture.

V.2. EFFECT OF GLUCOSE ON RAEC PROLIFERATION AND VIABILITY

HG may be expected to promote cellular growth and proliferation by supplying more energy *via* glycolysis. However, several studies have pointed out that HG infact decreases cell proliferation.

Experiments conducted on HUVECs have shown that HG decreases cell proliferation and promotes apoptosis [261]. The increase in population doubling time of RAECs exposed to HG, found in the present study is in agreement with previous reports on the effects of HG on endothelial cell proliferation [262, 263]. HG concentration of 35 mM was found to significantly affect viability of RAECs. This observation is in conformity with an earlier study which reported decreased viability of human aortic endothelial cells (HAECs) exposed to high glucose concentrations [264]. Reactive oxygen species (ROS) have been implicated in the HG mediated decreased cell proliferation and viability [261, 262]. Experiments were done on cells made quiescent by serum deprivation which compensated for the inhibitory effect of HG on cell proliferation. To avoid glucose toxicity to RAECs, a non-toxic 25 mM glucose concentration was used as HG treatment for the entire study.

V.3. EFFECT OF LOSARTAN, GENISTEIN AND SODIUM SALICYLATE ON HG INDUCED MCP-1 SYNTHESIS IN RAECs

V.3.A. Effect of losartan

Losartan is a strong non-peptide anti-hypertensive agent which primarily exerts its action by specific blockade of angiotensin II (Ang II) receptors [203]. Ang II is known to

increase MCP-1 synthesis in endothelial cells and this effect can be attenuated by losartan [204]. Flammer *et al.* suggested that losartan improved diabetes induced endothelial dysfunction through an anti-oxidative effect [207]. Therefore the effects of losartan itself in modulating HG induced MCP-1 synthesis in aortic endothelial cells independent of its Ang II receptor blockade was explored. Losartan did not inhibit HG induced MCP-1 synthesis in RAECs. This finding is in agreement with that of Chang *et al.* who found that losartan did not exhibit any anti-inflammatory effect on HUVECs [265]. Possibly, losartan do not have any anti-inflammatory and anti-oxidant action, independent of its Ang II receptor blockade effect.

V.3.B. Effect of genistein

Genistein was previously reported to inhibit secretion of MCP-1, ICAM-1 and VCAM-1 in activated human endothelial cells by modulating tyrosine kinase activity [219, 220]. Genistein was also found to completely block leptin-induced ROS generation, CPT-1 activation and MCP-1 synthesis in BAECs [218]. However the anti-oxidant and anti-inflammatory actions of genistein in endothelial cells exposed to various experimental conditions, which simulate diabetes conditions such as hyperglycemia is unknown. Therefore, the effect of genistein in modulating HG induced MCP-1 synthesis in aortic endothelial cells was explored. It was found that genistein could not inhibit HG induced MCP-1 synthesis in RAECs. I did not see any other reports that suggest anything contrary to what was observed in the present study. Hence it may be concluded that genistein do not have either an anti-inflammatory or anti-oxidant activity that can modulate HG induced MCP-1 synthesis in endothelial cells.

V.3.C. Effect of sodium salicylate

Sodium salicylate (NaSal) has been shown to inhibit p38 MAPK activity and superoxide generation in human coronary artery endothelial cells [242]. NaSal directly inhibits NFκB activation and the consequent expression of pro-inflammatory cytokines and mediators as well [243]. NaSal also causes uncoupling of oxidative phosphorylation which ultimately exerts an anti-inflammatory effect [244]. Despite the known property of NaSal in inhibiting NFκB activity and ROS generation, its anti-inflammatory effects on endothelial cells exposed to various stimuli that mimic diabetic conditions have not been investigated. Therefore, the effect of NaSal on HG induced MCP-1 expression in aortic endothelial cells was evaluated. NaSal did not inhibit HG induced MCP-1 synthesis in RAECs. As found out during the latter part of my study, MCP-1 regulation is not completely regulated by ROS or NFκB. Therefore even a complete blockade of ROS generation or NFκB activation by NaSal may not significantly attenuate HG induced MCP-1 synthesis. Another reason could be that NaSal is unable to modulate regulatory proteins other than NFκB involved in MCP-1 gene regulation. The effective dosage of NaSal that should have been used may be above 100 μM. The above observations suggest that NaSal may not have any modulating effect on HG induced MCP-1 synthesis in RAECs.

V.4. EFFECT OF CURCUMIN ON HG INDUCED MCP-1 SYNTHESIS IN RAECs

Curcumin is a polyphenolic compound with anti-oxidant and anti-inflammatory activity. It is also a known inhibitor of NFκB pathway [235]. The present study investigated whether curcumin would attenuate HG induced MCP-1 synthesis in RAECs and if so, what is the mechanism of curcumin action. The results indicate that curcumin attenuates HG induced MCP-1 gene expression in aortic endothelial cells can be attenuated by curcumin.

V.4.A. HG induces MCP-1 mRNA and protein synthesis in RAECs

The observation that there is increased MCP-1 protein synthesis in RAECs exposed to HG for 24 h is in conformity with earlier findings by others in human aortic endothelial cells (HAECs) [146, 186]. The amount of MCP-1 protein synthesized increases with the dose of glucose and duration of exposure of RAECs to HG levels and the effects are not due to osmolality. The time and dose dependent increase in MCP-1 synthesis is thought to depend on reactive oxygen species (ROS) accumulation in endothelial cells in a time and dose dependent manner [147]. ROS and p38 MAPK are both implicated in the HG induced MCP-1 expression in endothelial cells [7, 146, 147].

Even though several upstream signaling molecules such as ROS, p38 MAP kinases etc are reported to be activated upon HG induction, the final common pathway in HG induced MCP-1 expression in aortic endothelial cells is through NFκB pathway [7, 146, 186]. The upstream promoter sequence of MCP-1 gene contains two NFκB sites [202]. Therefore the involvement of NFκB in HG induction of MCP-1 induction by HG was investigated.

V.4.B. Curcumin inhibits HG induced NFκB nuclear translocation and DNA binding in RAECs

NFκB is sequestered in the cytoplasm by the regulatory proteins IκB and upon stimulation IκB is phosphorylated and degraded. Consequently NFκB is translocated into the nucleus where it binds to the consensus sequences mediating gene transcription [266]. Bay 11-7082 is a specific inhibitor of NFκB which prevents the phosphorylation of IκBα and the subsequent translocation of NFκB into the nucleus [197, 267]. In RAECs exposed to HG in the presence of Bay 11-7082, there was significant inhibition of HG induced MCP-1 mRNA synthesis suggesting involvement of NFκB in MCP-1 induction by HG. The present findings are in agreement with previous reports on the involvement of NFκB in HG induced MCP-1 expression in HAECs and human umbilical vein endothelial cells [7, 146, 186]. Similar to the effect of Bay 11-7082, curcumin also decreased HG induced MCP-1 mRNA synthesis in endothelial cells. Farhangkhoee *et al.* have earlier observed that curcumin inhibits NFκB in HG stimulated endothelial cells [240]. Hence it is reasonable to suggest that curcumin attenuates HG induced MCP-1 expression in aortic endothelial cells *via* its inhibitory effect on the NFκB pathway.

EMSA was done to confirm whether curcumin mediates its effect *via* NFκB. Similar to the effect of Bay 11-7082 in preventing NFκB DNA binding, curcumin also decreased NFκB DNA binding activity. The results obtained by confocal imaging of NFκB p65 nuclear translocation also supported the results obtained by EMSA which indicates that curcumin prevents HG induced translocation of p65 subunit from cytoplasm to nucleus. Accordingly it may be inferred attenuation of HG induced MCP-1 gene expression in

endothelial cells by curcumin is through blocking the nuclear translocation of NFκB and NFκB binding to the consensus sequences upstream of the MCP-1 promoter.

The fact that specific inhibitor of NFκB, Bay 11-7082 did not attenuate HG induced MCP-1 mRNA synthesis to the same extent as curcumin and the significant difference between the effect mediated by these two pharmacological inhibitors indicate a possible role for additional transcription factor / factors in the HG induced expression of MCP-1 in aortic endothelial cells. The transcription factor, Activating Protein-1(AP-1) is found to be activated in endothelial cells upon HG induction [7] and MCP-1 upstream promoter contains one AP-1 DNA binding site [202]. Studies that were taken out later have demonstrated inhibitory effect of curcumin on AP-1 DNA binding activity.

V.5. EFFECT OF QUERCETIN ON HG INDUCED MCP-1 SYNTHESIS IN RAECs

The present study demonstrates the anti-inflammatory and anti-oxidant activity of quercetin in glucose primed aortic endothelial cells. The study also reveals that quercetin mediated attenuation of HG induced MCP-1 expression in RAECs involves dual modulation of NFκB and AP-1 pathway and that the inhibition of MCP-1 expression is partly independent of quercetin's anti-oxidant activity.

V.5.A. Quercetin dose dependently attenuates HG induced MCP-1 protein synthesis in RAECs

There are only a few studies, which demonstrate that quercetin attenuates MCP-1 expression in endothelial cells, the cell type relevant to vascular homeostasis and atherosclerosis. Tribolo *et al.* have demonstrated the inhibitory effect of quercetin on MCP-1 expression in LPS/TNFα stimulated HUVECs [268]. They, however, did not investigate the mechanism of quercetin action. To my knowledge, there is no study detailing the mechanism of quercetin action in endothelial cells exposed to conditions that simulate diabetic settings.

Similar to the effect of curcumin, quercetin also attenuated HG induced MCP-1 protein synthesis in a dose dependent manner. Several groups have demonstrated dose dependent inhibitory effects of quercetin on MCP-1 expression in TNFα stimulated human aortic smooth muscle cells (HASMCs) [269], anti-IgE activated mast cells [270] and IL-1 stimulated glomeruli cells [271]. The effective dose of quercetin to be used in inhibitory studies is controversial; various concentrations ranging from 2 to 50 μM have been suggested to be the effective dose [210, 268]. In my study, 100 μM concentration was used for the entire experiments because this concentration attenuated HG induced MCP-1 protein synthesis in RAECs more efficiently than the 50 μM concentration (79% in 100 μM and 42% in 50 μM). Quercetin concentrations above 50 μM have been suggested to be pro-oxidant and toxic to endothelial cells [210, 268]. The viability of RAECs as assessed by MTT assay, was not affected at 100 μM concentration of quercetin (data is not shown). Lin *et al.* found that 100 μM quercetin in fact, protected human umbilical vein vascular endothelial cell line against homocysteine mediated injury by increasing

cell viability [272]. However I could observe significant loss of cell viability when semi-confluent RAECs were treated with 100 μM quercetin. Quercetin treatment at 100 μM was non-toxic to RAECs in the present experimental conditions, possibly because quercetin treatments were given to confluent RAEC cultures. Lotito and Frie found the IC_{50} for ICAM-1 in TNF α treated HAECs to be 50 μM quercetin [210]. In the present study, IC_{50} for MCP-1 in RAECs was found to be 50 μM quercetin. Therefore the use of quercetin at 100 μM in my study seems justifiable as it has maximum inhibitory effect with relatively minimum cytotoxicity.

V.5.B. Quercetin attenuates HG induced MCP-1 mRNA expression in RAECs and the effect is partly independent of ROS

The attenuating effect of quercetin on HG induced MCP-1 mRNA synthesis in RAECs was in conformity with similar effects of quercetin on MCP-1 expression in LPS-TNF α stimulated HUVECs [268] and TNF α stimulated HASMCs [269]. Comparative analysis of the levels of HG stimulated MCP-1 transcripts and proteins in RAECs reveal that the increase in MCP-1 mRNA and protein synthesis varies from 1.7 to 2 fold. Hence, HG stimulation of MCP-1 gene expression may be primarily regulated at the transcriptional level. The observation supports the hypothesis that transcription factors may be the key regulatory proteins involved in HG induced MCP-1 expression in RAECs.

In the current study, HG was found to increase ROS generation in RAECs and quercetin considerably inhibited HG induced ROS synthesis. The finding that quercetin inhibits ROS generation is in agreement with previous reports on the anti-oxidant activity of

quercetin in various cell types [210, 273-275]. The inhibition of ROS production in RAECs may be because of the direct scavenging of free radicals by quercetin. It is evident that the anti-oxidant activity of quercetin is not as effective as NAC, a specific inhibitor of ROS. The difference may be because of their different mode of action involving glutathione, a tripeptide antioxidant that protects cells from toxins such as free radicals [276]. Glutathione is found exclusively in reduced form and reacts with free radicals and gets converted to its oxidized form, glutathione disulfide. During oxidative stress the enzyme glutathione reductase gets activated by reverting the oxidized glutathione back to the reduced form [277]. Anti-oxidant action of quercetin involves direct neutralization of ROS in the cells and consequent oxidation to semiquinone and quinine. Quercetin does not directly enhance the effect of anti-oxidants such as glutathione and vitamins E and C but takes the help of anti-oxidants itself to regenerate both semiquinone and the quinine [278]. In HG stimulated RAECs, anti-oxidants such as glutathione may be in a depleted condition because of continued oxidative stress generated by HG. Quercetin may gradually become ineffective because of its own depletion and may not support the existing cellular anti-oxidants defence mechanism. NAC is a reduced thiol donor with non-specific antioxidant propertiesm that can support glutathione resynthesis [279, 280]. It can be assumed that in HG stimulated RAECs, NAC effects an indirect scavenging of free radicals through glutathione by converting oxidized glutathione back to the reduced form. Thus, NAC is a more effective anti-oxidant compared to quercetin because of the beneficial action of NAC on glutathione resynthesis. An additional reason for NAC to be a more effective anit-oxidant in the experimental conditions used in the present study settings present may be because of the

increased availability of NAC at a non-toxic concentrations (10 mM) compared to the concentration of quercetin (100 μM) in HG stimulated RAECs. Interestingly, compared to quercetin, NAC was found to be less effective in attenuating HG induced MCP-1 mRNA synthesis. Even though NAC effectively blocked ROS generation in HG induced RAECs, the same effect was not seen in HG induced MCP-1 mRNA synthesis. Possibly, HG induced MCP-1 synthesis in RAECs is not entirely dependent on ROS and there may be other active mechanisms such as PKC activation, polyol pathway etc. causing increased MCP-1 expression in HG induced RAECs.

Intracellular signal transduction events initiated by HG involves a variety of kinases such as PKC, MAPK, PKB etc. [192, 281, 282] which can activate specific transcription factors, eventually altering MCP-1 gene expression. Several studies have revealed that the regulation of MCP-1 gene in endothelial cells involves transcription factors namely NFκB and AP-1 [146, 186, 198]. The differences in the induction and binding of these transcription factors to the MCP-1 gene promoter may be a critical regulatory step that causes the MCP-1 gene to express in specific cell types and under specific stimuli. Therefore, an attempt was made to delineate mechanism of quercetin action on the transcriptional control of MCP-1 gene in HG stimulated aortic endothelial cells.

V.5.C. Quercetin prevents p65 nuclear translocation in HG stimulated RAECs

NFκB subunit p65 is sequestered in the cytoplasm by the regulatory proteins IκBs and upon stimulation IκB gets phosphorylated and degraded. Consequently p65 translocates into the nucleus where it binds to the consensus sequences mediating gene transcription

[266, 283]. The observation that the levels of p65 subunit of NFκB were decreased in cytoplasm of HG stimulated RAECs is in conformity with previous reports on NFκB signaling pathway. The fact that quercetin reversed the decrease in cytoplasmic p65 levels induced by HG in RAECs suggests successful inhibition of HG induced p65 nuclear translocation. This finding is also supplemented by confocal imaging data for p65 nuclear translocation where quercetin was seen to completely block HG induced p65 nuclear translocation. The ROS inhibitor NAC even though caused some inhibition, did not completely reverse the decrease in HG induced p65 nuclear translocation. Careful analysis of confocal imaging data of NAC inhibition of HG stimulated p65 nuclear translocation also supplemented the result obtained by Western blotting. Rangan *et al.* in a study on proximal tubular epithelial cells, also found NAC at sub-toxic concentrations to be ineffective in preventing NFκB activation [284]. The failure of NAC to prevent nuclear translocation of p65 despite having an effective ROS scavenging ability strongly suggests that NFκB activation in HG stimulated RAECs is mediated primarily by mechanisms other than oxidative stress.

V.5.D. Quercetin prevents IκBα degradation in HG stimulated RAECs

In the canonical NFκB signaling pathway, the decreased cytoplasmic p65 levels directly correlates with decreased IκBα levels and the consequent increase in the nuclear localization of p65 [197]. Analysis of IκBα in the cytoplasm of RAECs after 24 h revealed same levels of IκBα in both HG treated cells and the control. In RAECs stimulated with HG, the IκBα levels may be coming back to the basal level after 24 h as in control. This assumption is supported by earlier studies which also found an initial

decrease in cytoplasmic IκBα levels after stimulation because of ubiquitination and degradation of IκBα. The basal levels of IκBα are quickly regained as in control by the increased resynthesis of IκBα by NFκB itself [285, 286].

In addition to the cytoplasmic sequestration of NFκB, IκBs play a key role in preventing NFκB DNA binding under basal conditions and in making NFκB -IκB complexes responsive to diverse stimuli [283]. IκBα, the most important among IκBs was initially thought to regulate NFκB pathway by preventing the nuclear import of NFκB-rel complexes but evidences suggest that IκBα maintains basal cytoplasmic localization of these complexes through the action of its strong N-terminal nuclear export sequences. Thus, active nuclear export rather than the blockade of nuclear import underlies cytoplasmic localization of NFκB by IκBα. In addition, transcription of IκBα itself is stimulated by NFκB which ultimately removes active NFκB from its cognate sites, and exports it back to the cytoplasm to establish an auto-regulatory negative feedback loop [285, 287]. In this context, the present finding that quercetin significantly increases the level of cytoplasmic IκBα assumes significance. Quercetin possibly enhances the auto-regulatory negative feedback loop that helps to increase IκBα synthesis and consequent nuclear export of p65 back to the cytoplasm in HG stimulated RAECs. The finding that quercetin increases IκBα in the cytoplasm of HG stimulated RAECs to levels much higher than that in unstimulated RAECs suggests that the inhibitory effect of quercetin on NFκB pathway may primarily be due to its effect of increasing cytoplasmic IκBα levels. The findings that quercetin inhibits NFκB pathway has greater significance because NFκB is implicated in the regulation of most of the inflammatory genes associated with atherosclerosis [120, 288, 289].

V.5.E. Quercetin attenuates NFκB DNA binding activity in HG stimulated RAECs

Quercetin was found to effectively decrease HG induced NFκB DNA binding activity in RAECs. The finding supports current views on mechanism of inhibition of p65 nuclear translocation in HG induced RAECs by quercetin. The results are also in agreement with findings by Choi *et al.* and Di Santo *et al.* They demonstrated quercetin to block DNA binding activity of NFκB in TNFα stimulated HUVECs [290, 291]. Supershift analysis with antibodies against p50, p65 and c-rel revealed that NFκB bands are composed of p50-p65 heterodimers. This complex of NFκB is suggested to be the most abundant and the most effective form for enhancing gene expression [286, 292].

As discussed earlier, I have demonstrated NFκB to be involved in the transcriptional induction of MCP-1 gene in HG stimulated RAECs. Curcumin was found to attenuate HG induced MCP-1 expression more efficiently than the specific inhibitor of NFκB, Bay 11-7082 suggesting that curcumin may be mediating its effect by additionally interfering with some other regulatory proteins. As such, the effect of curcumin on AP-1 requires scrutiny because it is also recognized as an effective inhibitor of AP-1 [293-297]. In the present study, curcumin was found to effectively block HG induced AP-1 DNA binding activity in RAECs suggesting an inhibitory effect of curcumin on AP-1 pathway in RAECs. This also suggests that curcumin may be attenuating HG induced MCP-1 expression in RAECs by modulating the AP-1 pathway in addition to NFκB. Hence the role AP-1 in HG induced MCP-1 expression was further explored.

V.5.F. Quercetin attenuates AP-1 DNA binding activity in HG stimulated RAECs

Analysis of AP-1 DNA binding activity revealed that HG increased DNA binding activity of AP-1 in RAECs. Quercetin could effectively block HG induced AP-1 DNA binding. Inhibitory effect of quercetin on AP-1 DNA binding has been reported earlier in IL-1 stimulated HUVECs [298] and linoleic acid activated porcine endothelial cells [275]. The present findings are novel as they are the first demonstration of modulation of AP-1 pathway by quercetin in endothelial cells exposed to HG. Analysis of AP-1 binding activity gave three distinct bands for AP-1. An interesting finding in EMSA was the decreased intensity of the middle band in HG stimulated cells when compared with unstimulated RAECs. This suggests inhibition of a particular AP-1 complex that is part of the middle band upon HG stimulation in RAECs. It is also possible that this AP-1 complex of the middle band may be having an inhibitory effect on AP-1 driven gene promoters under basal conditions. This study could not successfully identify the AP-1 dimer complex that is part of the middle band. Determining the subunit composition of the middle band may shed more light into AP-1 regulation in HG stimulated RAECs.

AP-1 proteins consist of a variety of homodimers and heterodimers including the members of the Fos, Jun and CREB/ATF families [299]. Among them c-fos, c-jun and fra-1 are reportedly active in aortic endothelial cells exposed to high glucose conditions [300]. Supershift analysis with antibodies against c–jun, c-fos and fra-1 subunits resulted in the abrogation of AP-1 bands suggesting the presence of these proteins. As no shift in AP-1 bands was observed with specific antibodies against AP-1 components, the exact subunit composition of each AP-1 bands could not be determined. Supershift analysis

revealed the AP-1 bands to be predominantly composed of c-jun and fra-1 subunits. The other subunit namely c-fos was however found to be less in all the three bands. Generally, jun-fos heterodimers are suggested to be involved in responses to various stimuli [299]. The findings in the present study suggest that c-fos does not seem to be actively involved in the AP-1 complex formation. A reason can be the shorter half-lives of c-fos protein and mRNA when compared to c-jun [299]. Further, c-jun has got complex regulatory mechanisms which help it to maintain higher levels in the cell upon stimulation. These include post-translational modification of pre-existing c-jun thereby making it active and simultaneous induction of c-jun gene expression [301]. In RAECs stimulated with HG there might be an upregulation of c-jun gene with simultaneous post-translational modification of pre-existing c-jun proteins causing an increased AP-1 activity. This argument, however has to be further validated.

Confocal imaging was done to test whether there is increased localization of c-jun in the nucleus of HG stimulated RAECs. The results confirm that HG induces an increased nuclear accumulation of c-jun in RAECs. The finding that quercetin significantly inhibited HG induced nuclear localization of c-jun supports the data obtained from EMSA where quercetin was demonstrated to prevent AP-1 DNA binding. Careful analysis of confocal imaging data for c-jun, reveals that NAC marginally decreases HG induced c-jun localization in RAECs suggesting only a minor role for oxidative stress in HG induced AP-1 activation.

V.5.G. Quercetin attenuates MCP-1 promoter and NFκB and AP-1 enhancer activities

EMSA efficiently measures NFκB and AP-1 DNA binding activity but changes in DNA binding cannot mirror the transcriptional activity of this complex. Therefore it is essential to explore activation of NFκB or AP-1 driven reporter genes to determine their ability to regulate gene expression. Experiments were conducted to determine the activity of both MCP-1 full length promoter and AP-1 and NFκB enhancers.

EA.hy 926 cells were chosen for the transfection studies because of its high transfection efficiency compared to RAECs. These cells have been reported to upregulate MCP-1 synthesis and increase ROS generation upon HG stimulation [190] as was similar to the HG effects on RAECs.

Analysis of promoter activity revealed that HG significantly increased the activity of MCP-1 promoter and that this induction is attenuated by quercetin. The MCP-1 full promoter construct used in this study contained the MCP-1 promoter of mouse with two putative NFκB and one AP-1 binding site. The 2.5 kb 5' flanking region of mouse MCP-1 promoter sequence cloned in this construct share > 80% homology with that of rat. The homology within the 100 bp surrounding the putative NFκB and AP-1 binding sites of both mouse and rat is nearly 97% [302]. The core sequences of the two NFκB and one AP-1 site are identical in both rat and mouse MCP-1 promoters. Therefore it may be assumed that HG induced activation of mouse MCP-1 promoter seen in the present study may produce the same effect in rat MCP-1 promoter as well. Shanmugam *et al.* demonstrated that MCP-1 promoter activation in HG stimulated THP-1 cells [303] was seen with concomitant increase in MCP-1 mRNA levels. As increased promoter activity

is prerequisite for increased mRNA synthesis, it can be reasonably concluded that the increased MCP-1 mRNA levels in HG treated RAECs may be secondary to increased MCP-1 promoter activity.

The presence of NFκB and AP-1 consensus binding sequences of rat MCP-1 promoter respectively in pNFκB Luc and pAP-1 Luc luciferase vectors make it NFκB and AP-1 driven. The observation that HG increased the expression of pAP-1 Luc and pNFκB Luc constructs suggest the ability of putative NFκB and AP-1 binding sites to transcriptionally activate promoters of luciferase reporter gene. The increase in HG induced luciferase gene expression does not reflect a direct effect of AP-1 and NFκB on HG stimulated MCP-1 promoter activity. Instead it suggests that in HG stimulated cells, NFκB and AP-1 consensus binding sequences have the capacity to enhance the activity of a given promoter. Sreenivasan *et al.* in HG stimulated HAECs demonstrated that activation of NFκB and AP-1 was involved in IL-8 expression [304]. Their results lend support to the indications in the present study on the involvement of NFκB and AP-1 in the activation of MCP-1 promoter.

Quercetin was found to significantly attenuate HG induced MCP-1 promoter activation as well as NFκB and AP-1 enhancer activation. The quercetin might therefore, be inhibiting MCP-1 full promoter activation by modulating NFκB and AP-1 enhancers. The data obtained by EMSA where quercetin was found to decrease NFκB and AP-1 DNA binding activity in HG stimulated RAECs lend support to the attenuating effect of quercetin on HG induced activation of NFκB and AP-1 enhancers.

V.6. LIMITATIONS OF THE STUDY

- As gene expression under a variety of stimuli is primarily regulated by a few set of specific transcription factors for a gene, I explored regulation of MCP-1 gene mainly at the transcriptional level. Even though different signal transduction pathways involving kinases such as PKC, MAP kinanses, PKA etc have been reported to be active in HG stimulated cells, the present study did not look into their role in HG induced MCP-1 expression. Therefore more studies have to be done to understand the precise signaling mechanisms involved in the HG induced MCP-1 gene expression. A few other inflammatory molecules involved in atherosclerosis such as VCAM-1, ICAM-1 and IL-8 was also not explored for their expression in endothelial cells upon HG stimulation.

- Site directed mutagenesis for NFκB and AP-1 sequences in the full promoter of MCP-1 gene was not done. Transfection studies using these mutants for NFκB and AP-1 would have precisely determined the role of each of these transcription factors in HG induced activation of MCP-1 gene.

- The effects of quercetin on HG induced signaling pathways, cell adhesion molecules and chemokines in endothelial cells were not explored. Delineation of quercetin effect on these molecules would have increased the relevance of quercetin as an anti-atherosclerotic molecule in type 2 diabetes.

V. SUMMARY AND CONCLUSIONS

VI.1. SUMMARY

The findings of the present study can be summarized as follows:

1. HG induces MCP-1 synthesis in cultured RAECs in a time and dose dependent manner and the effect is not due to osmolality.

2. High glucose concentrations decrease viability and proliferation of RAECs in culture and the effect is dose dependent.

3. Losartan, genistein and sodium salicylate did not inhibit HG induced MCP-1 synthesis in cultured RAECs.

4. HG increases NFκB and AP-1 DNA binding activity in cultured RAECs.

5. NFκB bands are composed of p65-p50 heterodimers whereas AP-1 bands are composed predominantly of c-jun and fra-1 subunits.

6. HG increases ROS generation but it is only partly responsible for HG induced MCP-1 gene expression in cultured RAECs.

7. HG causes activation of MCP-1 promoter and NFκB and AP-1 enhancers.

8. Curcumin attenuates HG induced MCP-1 mRNA and protein synthesis in cultured RAECs.

9. Curcumin also prevents nuclear translocation of p65 subunit and DNA binding activity of NFκB.

10. Quercetin inhibits HG induced MCP-1 mRNA and protein synthesis as well ROS generation in cultured RAECs.

11. In HG stimulated RAECs grown in culture, quercetin prevents nuclear localization of p65 and c-jun subunits of NFκB and AP-1 respectively.

12. Quercetin decreases NFκB and AP-1 DNA binding activity in HG stimulated RAECs in culture.

13. Quercetin attenuates HG induced activation of MCP-1 promoter.

14. Quercetin also decreases HG induced activation of NFκB and AP-1 enhancers.

VI.2. CONCLUSIONS:

The present study demonstrates for the first time that HG induced MCP-1 expression in aortic endothelial cells involves the cooperative action of NFκB and AP-1. The study reveals that curcumin attenuates HG induced MCP-1 expression in aortic endothelial cells and that this attenuation is at least in part mediated *via* NFκB pathway. As curcumin is able to regulate HG induced MCP-1 expression in aortic endothelial cells and given that curcumin also reduces hyperlipidemia, decreases ROS levels in cells isolated from patients with diabetes and prevents collagen cross-linking in diabetic animals further detailed *in vivo* studies are requisite to examine whether curcumin can be developed as a drug to abate the increased risk for atherosclerotic vascular disease in type 2 diabetes.

The present work is also the first extensive demonstration on the effects of quercetin in aortic endothelial cells exposed to HG. HG increases MCP-1 synthesis, ROS generation, NFκB and AP-1 DNA binding activity, MCP-1 full promoter activity and also the NFκB and AP-1 enhancer activity in RAECs. The observation that quercetin effectively attenuates all the above mechanisms suggests that the inhibitory action of quercetin on HG induced MCP-1 synthesis in RAECs primarily involves its dual modulating effects on NFκB and AP-1 pathway. The inhibitory effect of quercetin on HG induced MCP-1 expression also involves an inhibition of ROS generation by quercetin. Given the anti-inflammatory, anti-oxidant and anti-atherosclerotic effects of quercetin and also its inhibitory effects on MCP-1 expression and NFκB and AP-1 activity in HG stimulated cells, the efficacy quercetin as a candidate drug molecule in attenuating the risk for atherosclerosis needs to be further evaluated.

VI.3. FUTURE DIRECTIONS:

- Given the role of MCP-1 in atherogenesis and the findings from the present study that HG induces MCP-1 in aortic endothelial cells, the cell type relevant to atherosclerosis, further validation of MCP-1 as a novel therapeutic target against the increased risk for atherosclerosis in type 2 diabetes mellitus, is necessitated.

- The strong anti-oxidant and anti-inflammatory effects of quercetin coupled with the demonstration of inhibition of HG induced MCP-1 expression as well as NFκB and AP-1 activation by quercetin commend further detailed *in vivo* animal experiments to demonstrate the efficacy of quercetin in abating the increased risk for atherosclerosis in diabetes.

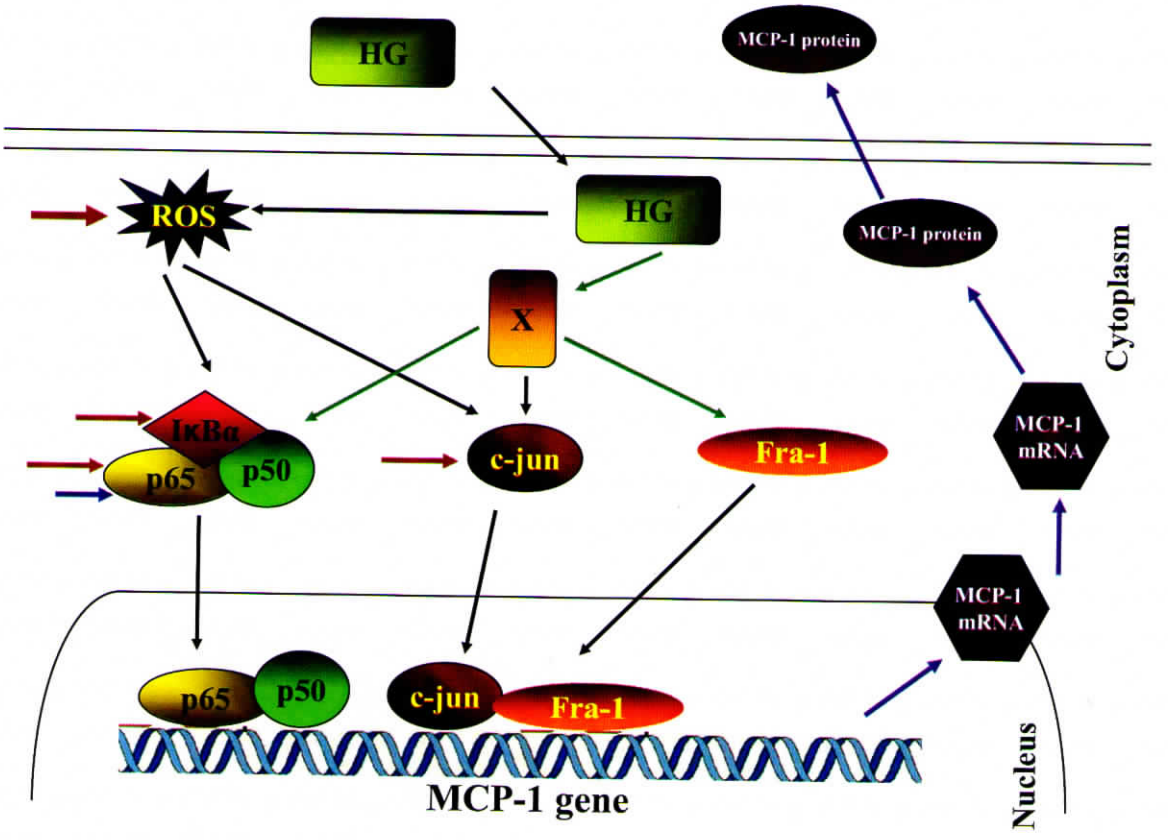

Figure 49: A diagrammatic model for HG induction of MCP-1 gene expression in aortic endothelial cells. Quercetin and curcumin attenuates HG induction of MCP-1 gene in aortic endothelial cells mainly by modulating NFκB and/or AP-1. '**X**' is the unknown mediator of HG induced signaling in aortic endothelial cells. The plausible sites of quercetin and curcumin action are indicated by arrows. [Quercetin in → and curcumin in →]

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VIII. LIST OF PUBLICATIONS

- **Panicker SR** and Kartha CC. **Curcumin Attenuates Glucose Induced Monocyte Chemoattractant Protein -1 Synthesis in Aortic Endothelial Cells by Modulating the Nuclear Factor κ B Pathway.** *Pharmacology* (2009, in press)

- Kaur S, **Panicker SR**, James T, Sarma PS, Thankappan KR and Kartha CC. **Association of MCP-1 -2518 polymorphism with metabolic syndrome in a south Indian cohort**. *Metab Syndr Relat Disord.* 2009 Jun;7(3):193-8

- **Panicker SR**, Babu MS and Kartha CC. **Quercetin attenuates high glucose induced MCP-1 expression in aortic endothelial cells by inhibiting nuclear factor-κB.** *Journal of Molecular and Cellular Cardiology*, 2008; 44:766-7. (Abstract)