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Hyperglycemia Induced Modulation of eNOS and ICAM-1 In Endothelial Cells by MEK5/ERK5/KLF2 Pathway

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Abstract

Aim: To investigate the role of Erk5 in hyperglycemia (HG)-induced Endothelial dysfunction (ED) via modulation of eNOS and ICAM-1 in primary Human umbilical vein endothelial cells (pHUVECs). **Methods:** pHUVECs were stimulated with different doses of glucose for different time intervals. MTT and Acridine Orange/ Ethidium Bromide (AO/EB) assays were done to examine cell viability and cytotoxicity. Western blotting to assess the protein expression and monocyte adhesion assay to determine monocyte adherence to pHUVECs and tube formation assay to determine endothelial characteristics were performed. **Results:** The results indicated that HG marginally decreased the viability of pHUVECs by 48 h of incubation and also decreased the expression of eNOS while increased that of ICAM-1. Incubation of pHUVECs with HG down regulated Erk-5 level, an indicator of compromised pHUVECs homeostasis. SiRNA-mediated silencing of Erk5 appeared to add to HG-mediated effects on eNOS and ICAM-1 as well as on monocyte adhesion. Further, HG decreased the tube formation ability of pHUVECs, which was again augmented when Erk5 was inhibited using synthetic inhibitor BIX02189. Furthermore, HG down regulated Mek5 and Klf2, the upstream and downstream molecules in Erk5 signalling. Inhibition of Erk5 further decreased Klf2 in HG-treated pHUVECs. **Conclusion:** In conclusion, HG down regulated Erk5 in pHUVECs causing ED by significantly decreasing eNOS and increasing ICAM-1 levels, which were phenotypically observed as increased monocytes adhesions and decreased tube formation ability of pHUVECs. These effects were likely regulated by the Mek5/Erk5/Klf2 pathway.

Keywords
Endothelial dysfunction (ED), Hyperglycemia, Erk5, eNOS, Endothelial homeostasis, Atherosclerosis.

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INTRODUCTION

Cardiovascular diseases (CVDs) are the leading causes of mortality and morbidity worldwide. Diabetes is one of the prominent risks for developing cardiovascular complications. In India, about 8.8% population is diabetic and the mortality rate due to CVDs is 24.8%, suggesting a strong correlation

between the two morbidities [1, 2]. Studies have shown a well-defined association between diabetes and CVDs, where diabetic patients suffer from vascular problems such as atherosclerosis, high blood pressure, stroke and vessel hardening[3].

Diabetic patients, especially type-2 diabetics, have a greater risk of developing atherosclerotic plaque compared to non-diabetic patients [4]. The primary reason for the initiation of atherosclerosis in diabetics is dysfunction of endothelial cells (ECs), the cells that line the inner side of blood vessels [5]. Hyperglycemia (HG) increases inflammation, expression of leukocyte adhesion molecules and leakiness of endothelium leading to a cascade of events that result in plaque formation[6]. Thus, survival and homeostasis of ECs is crucial for disease prevention during HG.

Recently, extra-cellular regulated kinase (Erk) 5, a transcriptional activator belonging to the MAPK family, has been identified and implicated in maintaining ECs homeostasis[7]. Studies have shown that inhibition of Erk5 is lethal to the organism due to failure in the development of cardiovascular system. Various studies have implicated the role of Erk5 in in pathophysiology of CVDs [8, 9].

Endothelial nitric oxide synthase (eNOS) is known to maintain the vascular tone by producing nitric oxide (NO) [10]. It is also important for ECs function and survival. Studies have suggested that deficiency of eNOS, which has been reported during HG, is associated with increased risk of CVDs [11]. Even though HG-induced damage is reported mostly through increased reactive oxygen species (ROS) production and downstream mediator kinases such as p38, JNK and AP-1 activation [12-14], the role of Erk5 in HG- Induced endothelial dysfunction (ED) is not clearly known. Intracellular adhesion molecule(ICAM)-1, belonging to the immunoglobulin super family, is constitutively expressed on the EC surface, it gets over expressed when exposed to HG or various pro-inflammatory cytokines[15]. Indeed, ICAM-1 is found to be significantly up regulated in ECs present at sites prone to atherosclerosis [16]. Several molecular mechanisms are activated during HG that regulate ICAM-1 and eNOS expression and contribute towards plaque development, however, the role of Erk5 is still elusive.

Erk-5 has been majorly studied with respect to shear stress generated in the blood vessels during high blood pressure however, understanding of its role during HG is limited. Therefore, we have investigated the role of Erk5 and its molecular mechanisms during HG that regulate the expression of ED markers such as eNOS and ICAM-1 and promote ED.

MATERIALS AND METHODS

Chemicals and Reagents

Antibodies against Erk5, ICAM-1 and GAPDH were purchased from Cell Signaling Technology (Denver, CO, USA), eNOS from BD Biosciences (San Jose, CA, USA), Krüppel-like Factor 2 (Klf2) from Thermo-Fisher Scientific (Waltham, MA, USA) and Mek5 from Millipore (Burlington, MA, USA). HRP conjugated anti-mouse or anti-rabbit secondary antibodies were procured from BioRad (Hercules, CA, USA). Dimethyl sulfoxide (DMSO), p-Coumaric acid from Sigma Aldrich (St. Louis, Missouri, USA), luminol and MTT dye from SRL (Mumbai, MH, India), D-glucose from HiMedia Laboratories (Mumbai, MH, India) and BIX02189 from Cayman chemicals (Ann Arbor, MI, USA) were procured. Specific siRNA for silencing Erk-5 (Cat. no. SI03024924) or scrambled siRNA and transfection reagent were purchased from Qiagen (Hilden, Germany).

Cell Culture and Treatment

Primary Human Umbilical Vein Endothelial Cells (pHUVECs) and its proprietary media and sub-culture reagents were purchased from Lonza (Basel, Switzerland). The cells (pHUVECs) were grown in optimum cell culture environment with 5% CO_2 with 95% humidity at 37°C in the CO_2 incubator (ESCO Micro PTE. LTD., Changi, Singapore). All the cellular experiments were performed at passages between 4 to 6.

MTT Assay

Approximately 10 x 10^3 pHUVECs/well were seeded in a 96-well plate and allowed to attach to the plate overnight. Cells were serum-starved overnight by incubating in 0.5% serum containing media and stimulated with different doses of glucose for 24 and 48 h in a final volume of 100 µl. At the end of incubations, 10 µl of 5 mg/ml of MTT dye was added to each well and incubated for 3 h. At the completion of incubation, media were aspirated, and the formazan crystals were dissolved in 100µL of DMSO. Absorbance was measured at 570nM using synergy H1 Hybrid micro plate reader (Winooski, VT, USA)

Acridine Orange/Ethidium Bromide (AO/EB) assay

Approximately 5 x 10^4 pHUVECs/well were seeded in a 24-well plate and allowed to adhere to the plate surface. Cells were starved as described earlier and treated with 25 mM glucose and incubated for 48 h. After incubation was over, media along with trypsinized cells was collected in 1.5 ml centrifuge tubes and centrifuged (2500 rpm; 5 min). The supernatant was removed, pellet was re-suspended in 50 µl of 1X PBS. The 10 µl of cell suspension was mixed with 3 µl of AO/EB dye solution and smeared onto a glass slide and observed under fluorescent

microscope (Olympus BX53 model, Tokyo, Japan). Images were captured using a camera (ProgRes® C5 model of Jenoptik, Thuringia, Germany) attached to the microscope.

Western Blotting

Approximately 2 x 10^5 pHUVECs / well were seeded in a 6-well plate. The cells were stimulated with glucose (25 mM) and incubated for 48 h. After incubation was over, cells were harvested and collected in a 1.5 ml centrifuge tube. After a brief centrifugation, the cell pellet was lysed with cell lysis buffer (50 mM Tris, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 150 mM NaCl) containing protease and phosphatase inhibitors. Equal amount of protein was separated onto 10% SDS-PAGE gels. Separated proteins were transferred to PVDF membrane followed by blocking with 5% skim milk powder for 1h at RT and then incubation with primary antibody overnight at 4°C. This was followed by 2 h of incubation with HRP-conjugated secondary antibody and visualization using the ECL detection system. Fluorescence was captured on X-ray film. Densitometry of Western blot images was performed using alpha imager software (San Jose, CA, USA).

SiRNA transfection in pHUVECs

Approximately 6 x 10^4 pHUVECs/well were seeded in a 24-well plate and allowed to adhere for 24 h. Subsequently, 10 nmol of Erk5 specific siRNA was mixed in 100 µl of serum-free media by vortexing. To the above mixture 3 µl HiPerfect transfection reagent was mixed and added drop wise in the wells containing 100 µl serum-free fresh media. Cells were incubated for 3 h followed by addition of 400 µl complete media and incubated for 48 h. The Erk5 protein expression was assessed by Western blotting.

Monocyte Adhesion Assay

Approximately, 6 x 10^4 pHUVECs / well were seeded in a 24-well plate and stimulated with glucose for 48 h after they adhered and starved for overnight. Subsequently, equal number of monocytes (1x10^5U937 cells) were layered over pHUVECs and co-incubated for 6 h. The cells were then washed with 1X PBS and images were captured using bright-field microscope. Number of monocytes adhering to pHUVECs were counted per microscopic area for quantification.

Tube-formation assay

Approximately 1 x 10^5 pHUVECs/well were seeded in a 12-well plate and allowed to adhere for 24 h. The cells were serum starved and treated with HG in the presence or absence of Erk5 synthetic inhibitor BIX02189 for 24 h. Cells were then trypsinised and re-seeded (at 25 x 10^3 cells/well density) in complete medium containing HG or BIX02189 or both, in a matrigel coated 96-well plate. After 4 h of incubation, tube formation was observed at 100X magnification using a bright-field microscope (Zeiss primovert, Oberkochen, Germany) and images were captured using a digital camera (Canon, Tokyo, Japan) attached to the microscope.

Statistical Analysis

All the experiments were performed in triplicates. The data has been represented as mean ± standard deviation (SD). One-way ANOVA followed by student's t-test (using Graph pad prism software version 5) was performed for analyzing the data (control vs treated group). The *p* value less than 0.05 were considered statistically significant.

RESULTS

HG decreased the viability of pHUVECs

We first examined the effect of high glucose by incubating pHUVECs with different concentrations of glucose for different time intervals followed by MTT assay. As shown in Figure 1A (i), there was no significant change in cell viability after 24 h of treatment. However, by the end of 48 h, there was marginal decrease the viability of pHUVECs by 12%, 12% and 11% at the concentrations of 25, 35 and 50 mM, respectively ($p<0.005$) (Figure 1A (ii)), even though no change in cell viability at 15 mM glucose concentration at both time intervals was observed. Based on these observations we chose 25 mM dose of glucose (defined as hyperglycemia or HG) for further experiments. We next performed AO/EB assay to further assess the effect of HG on pHUVECs viability and observed that 25 mM glucose caused cell death in pHUVECs (Figure 1B).

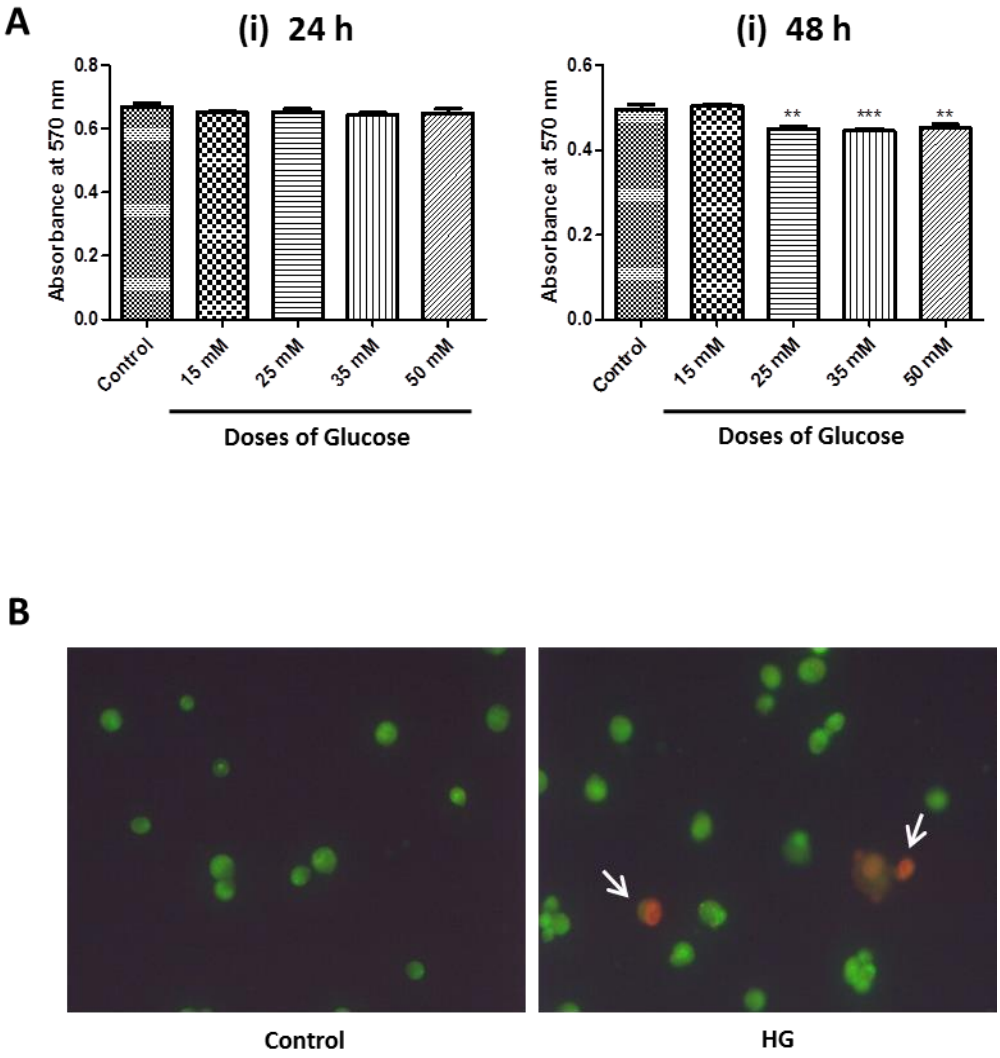

Figure 1: HG decreased the pHUVECs viability: (A) pHUVECs were stimulated with different doses of glucose for 24 and 48 h and MTT assay was performed. The bar shows mean ±SD, for 24 h **(i)** and for 48 h **(ii)** (n=4). **(B)** pHUVECs were stimulated with 25 mM of glucose for 48 and AO/EB assay was performed (n=3) and images were captured at 200X magnification. Arrow indicates the dead cells. ***p<0.0005; **p<0.005

HG decreased the expression of eNOS and increased that of ICAM-1 in pHUVECs

expression of eNOS which decreased to 0.1-fold (p<0.05). Similarly, ICAM-1 expression increased

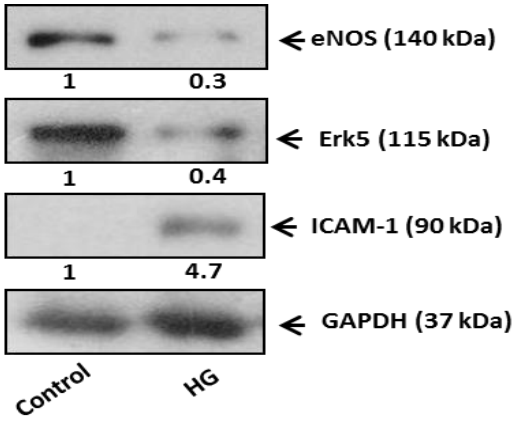

Figure 2: HG decreased the expression of eNOS and Erk5 and increased that of ICAM-1 in pHUVECs: Representative Western blotting image of eNOS, Erk5 and ICAM-1 in pHUVECs treated with HG for 48 h are shown (n=3). GAPDH blot shows loading control. Numbers below the blots show fold change.

We next examined the effect of HG on the expression of ECs marker eNOS, inflammatory marker ICAM-1 and the Erk5. As shown in figure 2, the expression of eNOS decreased to 0.3-fold (p<0.005) after HG treatment compared to control, the expression of ICAM-1 increased to 4.7-fold (p<0.05) in HG-treated pHUVECs compared to control. Further, the expression of Erk5 in HG treated cells decreased to 0.4-fold (p<0.05) when compared to control. These results suggested that HG decreased the levels of eNOS and Erk5 while increased that of ICAM-1, which could be responsible of HG-induced decrease in pHUVECs viability.

Erk5 silencing augmented the effects of HG in pHUVECs

Since changes in eNOS and ICAM-1 were coincidental with that of Erk5, we next examined whether eNOS and ICAM-1 were regulated by Erk5. We knockdown the Erk5 using siRNA-mediated silencing in pHUVECs and treated with HG. We observed that Erk5 silencing caused decrease in the expression of eNOS to 0.3-fold (p<0.05) whereas increased the expression of ICAM-1 to 4-fold (p<0.05) without any stimulation (Figure 3A). Further, HG treatment of Erk5 silenced cells caused additional decrease in the further to 4.8-fold (p<0.05) (Figure 3A). Additionally, we performed monocytes adhesion assay to examine the effect of silencing of Erk5 during HG. We observed that monocyte adherence increased after Erk5 silencing to 4.1-fold (p<0.0005) compared to control which was further increased to 6.4-fold (p<0.0005) when Erk5 silenced pHUVECs were treated with HG (Figure 3B).

Angiogenesis or tube formation is a primal property of ECs. Thus, we further examined the effect of Erk5 inhibition using its synthetic inhibitor BIX02189 on tube formation ability of pHUVECs. We observed that HG treatment caused significant inhibition of tube formation by 69.38% (p<0.0005). Erk5 inhibition alone also caused decrease in tube formation ability by 81.62%. However, when Erk5 inhibited cells were treated with HG, tube formation was inhibited by 90.81% (p<0.0005) suggesting additive Effect (Figure 3C).

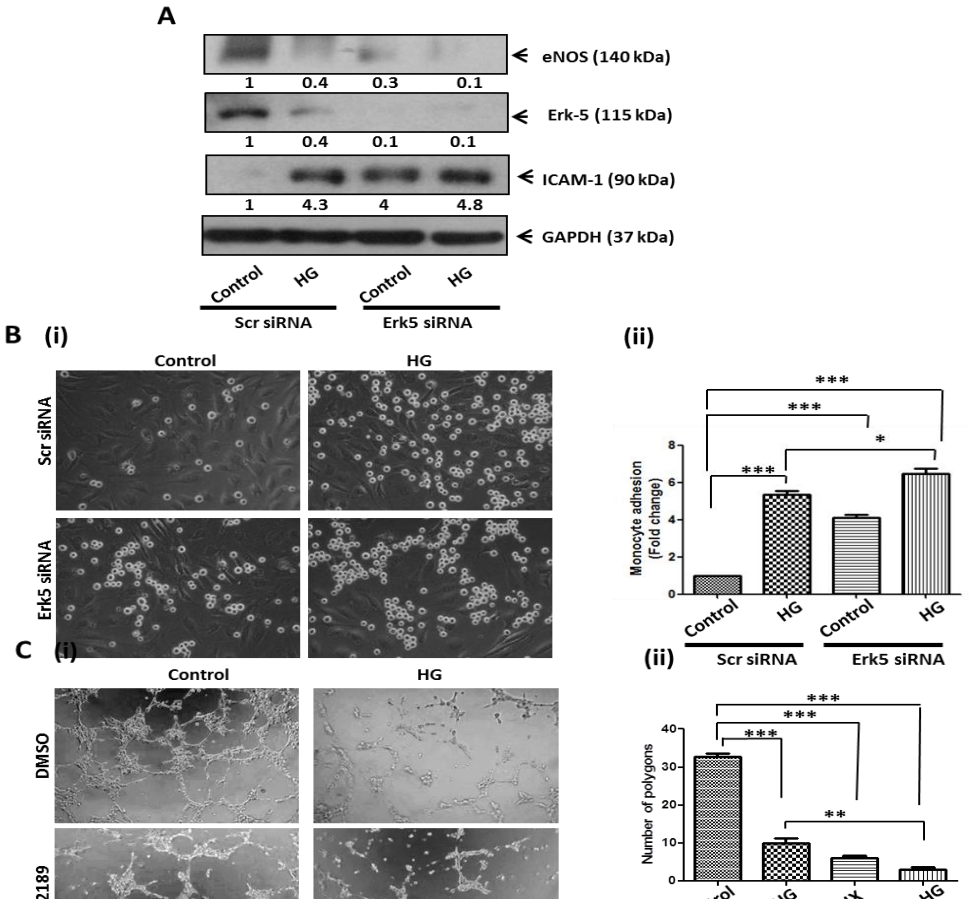

Figure 3: ERK-5 silencing augmented the effect of HG in pHUVECs: (A) Representative Western blot images (n=3) of eNOS, ICAM-1 and Erk5 in pHUEVCs after silencing Erk5 using siRNA with or without HG treatment are shown (n=3). GAPDH blot shows loading control. Numbers below the blots show fold change. **(Bi)** Monocyte adhesion to pHUVECs inErk5 silenced pHUVECs treated with or without HG for 48 h are shown **(Bii)** Bar diagram showing quantification (fold change) of monocyte adhesion Bar shows mean±SD, (n=3). **(Ci)** Representative images of tube formation assay in Erk5 inhibited pHUVECs using BIX02189 and treated with or without HG, **(ii)** bar diagram shows quantified data of tube formation by counting the number of polygons in at least 5 viewing areas per sample, mean±SD (n=3). ***p<0.0005; **p<0.005, *p<0.05

HG altered upstream and downstream mediators of Erk5 in pHUVECs

Since HG-induced decrease in Erk5 modulated the important EC marker eNOS, inflammatory marker ICAM-1 as well as monocyte adherence and tube formation, we next examined the modulators of Erk5 in pHUVECs under HG condition. We found that expression of Mek5, the upstream molecule of Erk5, was down regulated to 0.7-fold (p<0.05) while expression of Klf2, a downstream effector molecule of Erk5, decreased to 0.5-fold (p<0.05) when pHUVECs were treated with HG (Figure 4). Further, inhibition of Erk5 alone caused significant decrease in Mek5 and Klf2 expression to 0.5- and 0.3-fold (p<0.05). While treatment with HG did not further decrease Mek5 levels, the expression of Klf2 further decreased to 0.2-fold (p<0.05) when Erk5 inhibited cells were treated with HG. These results indicated that Erk5 inhibition alone was enough for down regulation of the levels of it signaling proteins, onslaught of HG further deteriorated the signals which are crucial for pHUVECs survival.

DISCUSSION:

Approximately 2.8% of population worldwide was diabetic in the year 2000 which is expected to rise to nearly 4.4% by 2030 [17]. According to the recent data India is the second largest sufferer of diabetes between the age group of 20-79 years after China with around 65.1 million diabetic patients[18]. The incidence of diabetes is expected to increase up to 109 million by 2035 A.D. Studies indicate that individuals with diabetes have 2- to 4-fold higher risk of developing CVDs [19]. Based on these statistics, it is of utmost importance to explore diabetes-associated cardiovascular complications, especially

atherosclerotic plaque development, which is the main cause of deaths in CVDs.

According to the recent studies Erk5, a recently identified member of the MAPK family, is associated with the regulation of cell proliferation, cell survival and cell differentiation [20, 21]. In the presence of various stressors such as shear stress, oxidative stress and osmotic shock Erk-5 activation is triggered by ECs to combat the adversity [22], however, its modulation is not clear in HG. In the present study we found that HG decreased the cell viability which was associated with decreased expression of Erk5.

The decreased expression of eNOS, which is known to catalyze the production of nitric oxide (NO), has been implicated in the decreased viability of ECs[23].

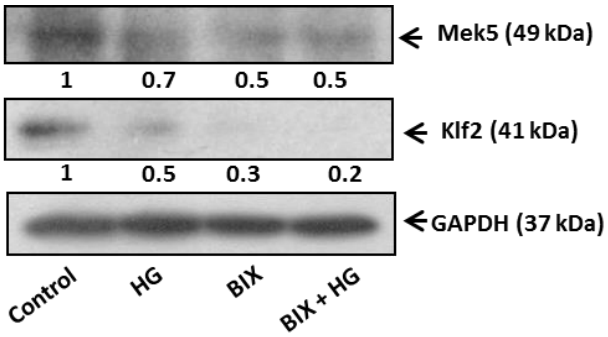

Figure 4: HG altered upstream and downstream mediators of Erk5: Representative image of Western blotting of Mek5, an upstream molecule and Klf2, a downstream mediator of Erk5 after treatment with Erk5 synthetic inhibitor BIX02189 in the presence or absence of HG (n=3). GAPDH blot shows loading control. Numbers below the blots show fold change.

The resultant low production of NO causes the loss of vascular tone and hardening of blood vessels. The eNOS activity in ECs is modulated by various circulating factors such as chemokines, inflammatory agents and has been identified to be regulated by various signaling pathways such as PI3K/AKT [24]. In our studies, HG decreased the expression of eNOS which may attributed towards the loss of cell viability. Further, siRNA-mediated silencing of Erk5 in pHUVECs results in reduction of eNOS expression. The effect was augmented when silenced cells were treated with HG. This indicated that Erk5 maintains eNOS expression and its decreased expression during HG could lead to decreased expression of eNOS.

ECs in the presence of cytokines or other stressors such as TNFα, IL-1β and modified lipids undergoes series of changes characterized by enhanced permeability, increased leukocyte adhesion and expression of pro-inflammatory markers such as ICAM-1 and VCAM-1 [15]. ICAM-1 is a prominent adhesion marker expressed on ECs during inflammation and helps in adhesion of circulating monocytes. A similar scenario was observed in HG-stimulated pHUVECs, where the expression of ICAM-1 was observed to be up regulated, indicating increased inflammation. The siRNA-mediated silencing of Erk5 increased ICAM-1 expression and also the adhesion of monocytes, indicating the role of Erk-5 in regulating inflammation in ECs. Since, HG was shown to decrease in the expression of Erk5, HG treatment of Erk5 silenced cells showed augmented increase in ICAM-1 level as well as adhesion of monocytes. Further, tube formation is a characteristic feature of ECs and loss of tube formation ability of ECs indicates their dysfunction. In the present studies, HG treatment decreased the tube formation ability in pHUVECs. Inhibiting Erk5 using synthetic inhibitor BIX02189 also decreased the tube formation, without HG treatment. The effect was enhanced when pHUVECs were treated with HG in the presence of BIX02189. These results indicated that Erk5 could be a protective molecule in ECs and HG-mediated down regulation of Erk5 results in alteration of eNOS, ICAM-1 and endothelial characteristics.

A number of stimuli such as cytokines, growth factors and oxidative stress are known to activate Mek5/Erk5 signaling in ECs and decreased Mek5/Erk5 signalling is linked with atherosclerosis and CVDs [21]. Mek5 lies upstream of Erk5, which binds and activates Erk5 by phosphorylation. Our study demonstrated that HG decreased the expression of Mek5. As Mek5 is exclusively essential for Erk5 activation, HG-induced decrease in Mek5

expression may lead to decreased levels of Erk5, which may further result in decreased eNOS expression as also observed in our studies. Further, Klf2, an anti-inflammatory protein and also a downstream mediator of Erk5 signalling, is known to up regulate eNOS and down regulate ICAM-1 expression in ECs. In the present study Klf2 expression was found to be decreased after HG treatment, which may be responsible for down regulation of eNOS and up regulation of ICAM-1 in HG-stimulated pHUVECs. Further, inhibition of Erk5 with its synthetic inhibitor led to decreased expression of Klf2, which indicates that Erk5 regulates Klf2 expression.

CONCLUSION:

Taken together, the present study indicates that Erk5 is crucial for maintaining ECs characteristics and homeostasis, which is hampered in the presence of HG leading to loss of ECs viability. HG affects endothelial cell viability by decreasing eNOS expression and increasing ICAM-1 expression via down regulation of Mek5/Erk5/Klf2 pathway, which could lead to ED. The present study thus identifies that Erk5 could be a molecular target in prevention of ED and atherosclerotic plaque formation during diabetic condition.

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CONFLICT OF INTEREST:

None

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