Glucose Regulates Monocyte Adhesion Through Endothelial Production of Interleukin-8

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Abstract—We have shown that glucose increases monocyte adhesion to human aortic endothelial cells (HAECs) in vitro. In the present study, we examined mechanisms by which glucose stimulates monocyte:endothelial interactions. HAECs cultured for 7 days in 25 mmol/L glucose had a 2-fold elevation in interleukin-8 (IL-8) secretion over control cells cultured in 5.5 mmol/L glucose (P<0.001). Use of a neutralizing antibody to IL-8 prevented glucose-mediated monocyte adhesion. Both glucose and IL-8 activated \( \alpha \beta \) integrin on the HAEC surface, suggesting that both activate the \( \alpha \beta \) integrin complex on the endothelial surface. The \( \alpha \beta \) integrin complex is important for anchoring connecting segment-1 fibronectin on the HAEC surface for monocyte adhesion. Analysis of the human IL-8 promoter revealed binding sites for NF-\( \kappa \)B and AP-1 as well as several aligned carbohydrate response elements (also known as E-boxes). Glucose dramatically stimulated IL-8 promoter activity. Using mutated IL-8 promoter constructs and EMSA, we found that the AP-1 element and the glucose-response element were responsible for much of the glucose-mediated activation of IL-8 transcription. Interestingly, inhibition of reactive oxygen species (ROS) production through use of pharmacological uncouplers of the mitochondrial electron transport chain significantly reduced glucose-mediated induction of IL-8 expression. These data indicate that glucose regulates monocyte:endothelial interactions through stimulation of IL-8 and ROS production and activation of the \( \alpha \beta \) integrin complex on HAECs. (Circ Res. 2003;92:371-377.)

Key Words: interleukin-8 • diabetes • endothelium • AP-1 • carbohydrate response element
appeared to be mediated through activation of the glucose response element and activation of the transcription factor AP-1. Increased endothelial production of IL-8 accelerates monocyte adhesion to endothelium. The activation of IL-8 by glucose is a primary mechanism by which hyperglycemia contributes to the accelerated vascular disease that occurs in diabetes.

Materials and Methods

Reagents

Fetal bovine serum was obtained from HyClone. ELISA reagents for human IL-8 were purchased from Endogen. Mitochondrial ROS inhibitors carbonyl cyanide m-chlorophenylhydrazone and thenoyltrifluoroacetone were purchased from Sigma. Calcine AM was purchased from Molecular Probes. Taqman probes and primers for human IL-8 and human GAPDH were purchased from Perkin Elmer. The neutralizing antibody for human IL-8 (AF-208-NA) was purchased from R&D Systems. Lipofectin was purchased from Invitrogen. HUTS-21 antibody was directed against the active conformation of β integrin was purchased from Pharmingen. NF-κB (No. E3291) and AP-1 (No. E3201) oligonucleotides were purchased from Promega.

Cell Culture

Human aortic endothelial cells (HAECs) were obtained from aortic rings of explanted donor hearts. HAECs were cultured for 7 days in Medium 199 containing 20% heat-inactivated FBS, 20 μg/mL ECGS, and 90 μg/mL heparin in the presence of 5.5 mmol/L glucose (NG) or 25 mmol/L glucose (HG) for 7 days. The 7-day, 25 mmol/L HG incubation condition was chosen because monocyte adhesion to endothelial cells was maximal at this concentration of glucose and time of incubation. For studies using chemical uncouplers of mitochondrial function, HAECs were described as above and treated for 7 days with 0.5 μmol/L carboxyl cyanide m-chlorophenylhydrazone or 10 μmol/L thenoyltrifluoroacetone.

For culture of porcine aortic endothelial cells (PAECs) for transient transfection studies, aorta was removed from male Yorkshire pigs aged 26 to 30 weeks fed a normal chow diet. Pigs were euthanized according to guidelines approved by the American Veterinary Medical Association Panel on Euthanasia and the University of Virginia. Pigs were obtained from Dr Ross Gerrity, Medical College of Georgia, Augusta, Ga. Aortas were collected in ice-cold M199. Endothelial cells were scraped gently from the aorta using a sterile cell scrapper and collected in M199 supplemented with 20% heat-inactivated FBS, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 30 μg/mL of ECGS. PAECs were plated into 1% gelatinized flasks and used from passages 3 to 6.

Human Monocyte Adhesion Assay

Our laboratory has recently developed a monocyte adhesion assay that utilizes primary mouse aortic endothelial cells and WEHI 78/24 cells. WEHI 78/24 cells are a mouse monocytoid cell line that has been fully characterized by McEvoy and colleagues. Our laboratory has recently developed a monocyte adhesion assay that utilizes primary mouse aortic endothelial cells and WEHI 78/24 cells. WEHI cells were labeled with calcein AM as described by the manufacturer. MAECs were incubated with 35 000 calcein-labeled WEHI cells/well for 30 minutes at 37°C. Nonadherent cells were rinsed, and the cells fixed with 1% glutaraldehyde. The number of attached monocytes within a 10×10 eyepiece grid was counted using fluorescent microscopy.

Promoter Studies

The human IL-8 promoter-reporter construct contained 1481 bp of the human IL-8 promoter. Plasmid constructs of the human IL-8 promoter containing a mutated NF-κB site or an mutated AP-1 site were generated as described previously. The NF-κB element was mutated from TGAATTTCT to TGGAATTTAaa. The AP-1 element was mutated from TGACTCA to TGACTGtt. For transient transfections, MAECs were grown in 5.5 mmol/L (NG) or 25 mmol/L (HG) glucose for 7 days on gelatin-coated plates as described above. MAECs were utilized in these transfection studies because transient transfection of primary HAECs is quite difficult. Transient transfection rates of primary HAECs were less than 10% of cells, yet transfection rates of primary PAECs were found to be 25% to 30% of cells (data not shown). Also, we have found that PAECs responded in a similar manner to glucose as did HAECs. Thus, we utilized PAECs in the transfection studies. PAECs were transfected in 12-well plates with 2 μg plasmid DNA using Lipofectin. TNF-α (10 U/mL) was incubated with the cells for 4 hours before harvest as a positive control for IL-8 activation. Cells were harvested for luciferase activity using a Reporter Lysis kit (Promega) at 24 hours after transfection. Luminescence was analyzed on a Turner Designs, Inc, luminometer. Luminescence was normalized to total cell protein.
Figure 1. Monocyte adhesion is increased to endothelial cells from diabetic mice. Endothelial cells were isolated from aorta of C57BL/6J (CTR) and diabetic (db/db) mice. Cells were used from passages 3 to 6. Adhesion assays using WEHI cells, a mouse monocyte cell line, were performed as described in Materials and Methods. *Significantly higher than CTR, P<0.001. Data represent the mean±SE of 5 experiments.

Results

Monocyte Adhesion to Endothelial Cells Is Increased in Diabetic Mice

Mice that have a defect in the leptin receptor (designated db/db) are hyperglycemic and insulin resistant as early as 6 weeks of age. At 6 to 12 weeks of age, these mice are used as a model of type 2 diabetes. We recently have developed a technique for isolation of primary endothelial cells from mouse aorta. Using this approach, we examined monocyte adhesion to endothelial cells from control (C57BL/6J) and diabetic (db/db) mice. As shown in Figure 1, we found that basal, unstimulated ECs isolated from db/db mice bound more monocytes than did C57BL/6J control ECs in a static adhesion assay (P<0.001). These data suggest that db/db mouse endothelial cells are already "preactivated" to bind monocytes, and indicate that monocyte adhesion to endothelium is increased in the diabetic state.

We have previously shown increased adhesion of monocytes to endothelial cells that had been cultured for 7 days in 25 mmol/L glucose. A dose-response curve of monocyte adhesion in response to glucose indicated a stepwise increase in monocyte adhesion to HAECs cultured in 25 mmol/L, 30 mmol/L, and 50 mmol/L glucose (data not shown). No significant increases in monocyte adhesion were observed at glucose concentrations below 25 mmol/L. Furthermore, a time course of incubation of HAECs in 25 mmol/L glucose indicated that monocyte adhesion significantly increased after 4-day incubation in glucose, and adhesion was maximal at 7 days (data not shown). There was no significant increase in adhesion to HAECs cultured for less than 4 days in 25 mmol/L glucose.

Glucose Regulates IL-8 Production in Endothelial Cells

Our hypothesis is that glucose activation of HAECs triggers production of chemokines that modulate monocyte recruitment and adhesion to endothelium. For these studies, we used HAECs that had been cultured for 7 days in 25 mmol/L glucose based on our findings described above. Two of the chemokines involved in mediating monocyte recruitment are IL-8 and RANTES. Levels of RANTES were not changed by glucose (data not shown). However, we observed dramatic elevations in levels of IL-8 mRNA (see online Figure 1, available in the online data supplement at http://www.circresaha.org) and observed a 2-fold increase in IL-8 secretion by endothelial cells in response to glucose (Figure 2).

Role of IL-8 in Mediating Monocyte:Endothelial Interactions in Response to Glucose

To determine the role of IL-8 in mediating monocyte adhesion, two experiments were performed. First, HAECs were incubated for 30 minutes with different concentrations of recombinant human IL-8 before addition of monocytes. IL-8 at 5 ng/mL maximally stimulated monocyte adhesion to HAECs (data not shown). This concentration of 5 ng/mL is well within the range secreted by HG-cultured ECs (see Figure 2). Secondly, HG-cultured HAECs were incubated with a neutralizing antibody to IL-8 before the addition of monocytes. Blocking IL-8 in HAECs completely blocked monocyte adhesion (Figure 3), indicating that IL-8 plays a key role in glucose-mediated monocyte adhesion.

We have previously shown that activation of HAECs by glucose promoted deposition of CS1 fibronectin on the EC surface. CS-1 is an adhesion molecule that can bind to the αvβ3 integrin complex on the EC surface. To determine the effects of glucose on β3 integrin activation, we used the monoclonal antibody HUTS-21. The HUTS-21 antibody recognizes only
A specific measure of MnCl₂ was used as a positive control to measure significantly higher than NG, P < 0.001; #significantly lower than HG, P < 0.008 by ANOVA. Data represent the mean ± SE of 3 experiments.

Regulation of Human IL-8 Promoter Activity by Glucose

To verify that the change observed in IL-8 mRNA in response to glucose was mediated at the level of mRNA, we performed studies in the presence of actinomycin D. Immediately after addition of actinomycin D, we incubated the cells for 24 hours in 25 mmol/L glucose. IL-8 mRNA levels were measured using real-time quantitative PCR. Addition of actinomycin D completely inhibited the HG-mediated increase in IL-8 mRNA (data not shown), confirming that glucose-mediated changes in IL-8 levels were regulated at the level of transcription.

To examine regulation of IL-8 promoter activity by glucose, we utilized a luciferase expression vector, pGL₂ Basic (Promega) that contained −1481 to +44 bp of the human IL-8 promoter. Analysis of the human IL-8 promoter using

**Figure 3.** Neutralizing antibody to IL-8 blocks monocyte adhesion. HAECs were cultured for 7 days in 5.5 mmol/L (NG), 25 mmol/L (HG) glucose, or 25 mmol/L L-glucose (L-Glu). Neutralizing antibody to IL-8 was incubated with HG cells (HG + IL8Ab; 20 μg/mL) for 2 hours before a monocyte adhesion assay. *Adhesion significantly higher than NG, P < 0.001; #significantly lower than HG, P < 0.01 by ANOVA. Data represent the mean ± SE of 5 experiments.

MacVector software (CGC, Inc) revealed binding sites for NF-κB and AP-1, as well as the glucose- or carbohydrate-response element as shown in the Table. 29–31,34 The human IL-8 promoter-reporter construct was transfected into primary PAECs. PAECs respond to glucose in a similar manner as HAECs and are much easier to transfect. 25 Incubation of PAEC with glucose for 4 hours only minimally stimulated IL-8 promoter activity, whereas cells cultured for several days in glucose displayed increased IL-8 promoter activity (Figure 5A). These data suggested the activation of additional cis- or trans-acting transcriptional element(s) by glucose. To study this hypothesis, we examined involvement of NF-κB and AP-1 promoter elements in glucose-mediated activation of IL-8. Using a human IL-8 promoter construct that contained a mutated NF-κB site, we still found significant activation of the promoter by glucose (Figure 5B). Using a human IL-8 promoter construct that contained a mutated AP-1 site, we found significant inhibition of IL-8 promoter activation by glucose (Figure 5B). These data indicate that AP-1 activation by glucose stimulates IL-8 production in HAECs. We confirmed these data using EMSA (Figure 6A), where we found minimal changes in NF-κB binding to endothelial nuclear extracts (NF-κB binding is significantly decreased) yet dramatic increases in AP-1 binding to endothelial nuclear extracts (Figure 6B).

We also examined binding of the glucose- or carbohydrate-response element (CHO-RE) to endothelial nuclear extracts from control and glucose-cultured cells. There was a significant increase in binding to the glucose response element in nuclear extracts from glucose-cultured HAECs compared with control (Figure 6B). Taken together, these data suggest that glucose activates multiple inflammatory or stress signaling pathways in HAECs. However, the primary regulators of IL-8 transcription in HAECs mediated by glucose appear to be AP-1 and CHO-RE. Oxidative stress activates AP-1-regulated pathways. 35

**Figure 4.** Glucose and IL-8 activate β₁ integrin on the endothelial surface. HAECs were cultured for 7 days in 5.5 mmol/L (NG) or 25 mmol/L (HG) glucose or incubated for 4 hours with 5 ng/mL recombinant human IL-8 (HuIL8). Cell-surface ELISA for activated β₁ integrin was performed as described in Materials and Methods. *Significantly higher than NG, P < 0.0001; #significantly higher than NG, P < 0.008 by ANOVA. Manganese chloride (MnCl₂) was used as a positive control to measure β₁ integrin activation. Data represent the mean ± SE of 3 experiments.
Atherosclerosis is a major risk factor of type 2 diabetes. Endothelial activation to bind monocytes is a key early event in these processes. This is the first report that shows glucose activation of IL-8 production in aortic endothelial cells and its link to monocyte:endothelial interactions. We show that EC chronically cultured in 25 mmol/L glucose for 7 days have increased production of IL-8. We chose this time and concentration of glucose in that this was the lowest concentration of glucose that gave maximal stimulation of monocyte adhesion in our assay. The increase in IL-8 production appeared to be regulated at the level of mRNA abundance, as IL-8 mRNA increased several-fold in response to glucose and this increase was sensitive to actinomycin D. In promoter-reporter studies, we found that glucose activated IL-8 through AP-1 and CHO-RE binding elements located within the human IL-8 promoter. The discovery that glucose activates endothelial cells to produce IL-8, which in turn, accelerates monocyte:endothelial interactions is an important and novel finding. This process may be a primary link between hyperglycemia and the mechanisms leading to atherosclerotic plaque formation.

Interestingly, IL-8 may have multiple roles in mediating monocyte:endothelial interactions. Firstly, as a secreted chemokine, it signals recruitment of monocytes. Previously, IL-8 was thought to play only a minor role in mediating monocyte recruitment and adhesion and was believed to be more closely associated with neutrophil chemotaxis. The studies of Gerszten and colleagues illustrated a new role for IL-8 in mediating monocyte rolling, and the recent elegant studies of Ley and colleagues implicated KC, the murine homolog of IL-8, as being the primary regulator of monocyte arrest in atherosclerotic carotid arteries. Ley and colleagues found that KC was more important than monocyte chemoattractant protein-1 (MCP-1) for mediating monocyte adhesion. Yeh and Berliner have recently shown that IL-8 is a mediator of oxidized phospholipid activation of monocyte

To further support the role of oxidative stress events in glucose-mediated IL-8 production, we used chemical uncouplers of the mitochondrial electron transport chain. Thenoyltrifluoroacetone (TTFA) inhibits Complex II of the electron transport chain and carbonyl cyanide m-cresolphenylhydrazone (CCCP) disrupts the proton gradient through uncoupling of mitochondrial oxidative phosphorylation. Both TTFA and CCCP block glucose-mediated ROS production (see online Figure 2). Importantly, these mitochondrial electron transport chain inhibitors also block IL-8 secretion in response to glucose (Figure 7B). These data indicate that activation of oxidative stress pathways generated by elevated glucose in endothelial cells leads to induction of IL-8 and increased monocyte:endothelial interactions.

Discussion

Figure 5. Glucose activates the human IL-8 promoter through activation of AP-1. PAECs were cultured in 5.5 mmol/L (NG) or 25 mmol/L glucose for 4 hours (HG-4h) or 7 days (HG-7d). A, PAECs were transfected with a plasmid containing −1481 to +44 bp of the human IL-8 promoter for 48 hours before measurement of luciferase activity. Luciferase activity was measured in a luminometer and was normalized to total cell protein. TNF−α (10 U/mL) and OxLDL (250 μg/mL) were used as positive controls for IL-8 promoter activation. *Significantly higher than NG, P<0.01 by ANOVA; #significantly higher than NG, P<0.0001 by ANOVA. B, PAECs were cultured for 7 days in 5.5 mmol/L (NG) or 25 mmol/L (HG) glucose and transfected for 48 hours with a luciferase reporter plasmid containing −1481 to +44 bp of the human IL-8 promoter. This promoter contained a mutated NFκB site (see Materials and Methods). Luciferase activity was normalized to total cell protein. OxLDL was used as a positive control to show IL-8 promoter activation in the absence of NFκB. *Significantly higher than NG by ANOVA, P<0.001. Data represent the mean±SE of 6 experiments performed in triplicate. C, PAECs were cultured for 7 days in 5.5 mmol/L (NG) or 25 mmol/L (HG) glucose and transfected for 48 hours with a luciferase reporter plasmid containing −1481 to +44 bp of the human IL-8 promoter. This promoter contained a mutated AP-1 site (see Materials and Methods). Luciferase activity was normalized to total cell protein. OxLDL and TNF−α were used as positive controls to show IL-8 promoter activation in the absence of AP-1. *Significantly higher than NG by ANOVA, P<0.0001; #significantly higher than NG by ANOVA, P<0.001. Data represent the mean±SE of 5 experiments performed in triplicate.

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adhesion to endothelial cells. Our data in Figure 3 implicates IL-8 as a primary mediator of monocyte:endothelial interactions. Secondly, IL-8 can also trigger activation of endothelial integrin (Figure 4). The endothelial integrin complex binds to CS-1 fibronectin, and CS-1 is a counter-receptor for VLA-4 on monocytes. We have previously shown that glucose upregulates CS-1 fibronectin deposition on the apical surface of aortic endothelial cells. IL-8 may contribute to adhesive events through activation of β1 on the endothelial cell surface. Taken together, these studies place IL-8 as the primary chemokine involved in mediating monocyte adhesion to activated endothelium.

IL-8 appeared to be activated by several signaling pathways. With regard to our studies of human IL-8 promoter activation by glucose, promoter activity was increased several-fold only after the cells had been cultured for 7 days in glucose. These data indicate the activation of secondary transcriptional element(s). As shown in Figures 5 and 6, NF-κB elements appeared to be responsible for only a small part of IL-8 promoter activation in response to glucose. Although NF-κB appeared to play some role in IL-8 promoter activation, ECs cultured in glucose showed less binding to a labeled NF-κB consensus sequence. These data suggest that glucose could be downregulating a repressor of the NF-κB response element. Or, it could be that NF-κB does not play a major role in human IL-8 promoter activation by glucose. Of particular novel interest are our findings of glucose-mediated activation of AP-1 and CHO-RE. AP-1 activation appeared to be largely responsible for glucose-mediated induction of IL-8 (Figure 5). AP-1 activation occurred by short-term treatment (4 hours; data not shown) and long-term treatment (7 days) in glucose (Figure 6). By gel shift assay, we found that the CHO-RE was activated by glucose at 7 days (Figure 6) and at 4 hours (data not shown). Studies to determine if the CHO-RE is important in IL-8 transcriptional activation still need to be performed; however, it is probable that this element will be important for IL-8 activation, in that the human IL-8 promoter contains multiple E-boxes (see Table).

Another exciting finding in our study is that inhibition of ROS production by glucose in endothelial cells reduced IL-8 production (Figure 7). These data suggest that a relationship exists between mitochondrial function and events leading to monocyte:endothelial interactions. The results shown in Figure 7 indicate that glucose modulates endothelial mitochondrial function. Glucose leads to an increased production of ROS by endothelial cells (see online Figure 2). The ROS activate several inflammatory pathways, including MAP kinases, NF-κB, and AP-1. Activation of these pathways stimulates IL-8 production, which leads to accelerated monocyte:endothelial interactions. We will continue to explore the pathways activated by glucose in endothelial cells, and will...

Figure 6. AP-1 and CHO-RE elements are important in glucose-mediated activation of IL-8 in endothelial cells. A, EMSA for NF-κB was performed in nuclear extracts from endothelial cells cultured in 5.5 mmol/L glucose (NG) or 7 days in 25 mmol/L glucose (HG) as described in Materials and Methods. Bands were supershifted by incubation of nuclear lysates with an antibody to p65 (SS). Pr indicates probe alone. *Significantly decreased from NG by Student’s t test, P<0.02. B, EMSA for AP-1 and CHO-RE were performed in nuclear extracts of endothelial cells cultured in 5.5 mmol/L glucose (NG) or 25 mmol/L glucose for 7 days (HG).

Figure 7. Mitochondrial ROS production is linked to IL-8 secretion. HAECs were cultured for 7 days in 5.5 mmol/L glucose (NG) or 25 mmol/L glucose (HG) in the presence of inhibitors of the mitochondrial electron transport chain (HG + TTFA and HG + CCCP). IL-8 secretion into HAEC media was measured by ELISA using antibodies specific for human IL-8. IL-8 secretion was normalized to total cell protein. #TTFA and CCCP significantly blocked IL-8 secretion, P<0.001 by ANOVA; *significantly higher than NG, P<0.0001. Samples were analyzed in triplicate.
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Additional Methods:

Monocyte adhesion assay: HAEC were cultured to confluency as described above in 48-well plates. Prior to the assay, HAEC were rinsed with 1% M199. Human primary monocytes were isolated from healthy normal volunteers using a modification of the Recalde method. After isolation, monocytes were labelled with Calcein AM for 15 mins at 37C. Labeled human primary monocytes (50,000/well) were added to HAEC monolayers, and incubated for 30 mins at 37C. Unbound monocytes were rinsed, cells were fixed in 1% glutaraldehyde, and bound labeled monocytes were counted within a 10x10 grid using epifluorescence microscopy. For experiments using the human IL8 neutralizing antibody, confluent endothelial cell monolayers were incubated with 20μg/ml of antibody for 2h at 37C. Cells were rinsed with media and incubated with labeled monocytes as described above for 30 minutes at 37C. The experimental use of HAEC and human monocytes was approved by the University of Virginia Institutional Review Board, and all procedures were performed in accordance with University guidelines.

Cell surface integrin ELISA: For measurement of activated β1 integrin on endothelial surfaces, we modified a cell-surface ELISA specific for the active conformation of β1 integrin. For this assay, HAEC cultured in NG or HG were cultured to confluency in 96 well plates. In some experiments, HAEC cultured in NG were treated with 5ng/ml recombinant human IL8 or with 6 mmol/L MnCl₂ (as a positive control for β1 integrin activation) for 2h at 37C prior to the assay. After incubation, cells were rinsed with 1XPBS containing Ca/Mg and were fixed in 4% paraformaldehyde for 20 min at rt. After fixing, cells were rinsed with 1xPBS+0.1%fatty acid free-BSA (0.1% FAFBSA). To block nonspecific binding, cells were incubated in 3% FAFBSA for 1h rt. Cells were then incubated for 2h at rt with 2 μg/ml HUTS-21 antibody. After primary antibody incubation, cells were rinsed and then incubated with secondary antibody (1:4000) for 1h.
at rt. Following incubation, cells were rinsed in 1XPBS followed by a rinse in double distilled H₂O. The peroxidase color reaction was developed using TMB Substrate solution (Pierce) and H₂SO₄. The plate was read on a Molecular Devices SpectraMax 190 spectrophotometer at a wavelength of 450 nm.

References:


Online Supplement Figure 1. IL8 mRNA is induced in HAEC by glucose. HAEC were incubated in 5.5 mM (NG), 25mM glucose (HG) for 7d, or 25mM L-glucose for 7d (L-Glu). Total cellular RNA was isolated from HAEC using Trizol, and genomic DNA was removed by DNAseI. RNA was used in quantitative RT-PCR using Taqman probes and primers for human IL8 mRNA and normalized to GAPDH as described in Methods. *IL8 mRNA was induced in HAEC cultured in HG, P<0.0001 by ANOVA.

Online Supplement Figure 2. Mitochondrial ROS production is increased in HAEC by glucose. HAEC were cultured for 7d in 5.5 mM glucose (NG) or 25mM glucose (HG) in the presence of inhibitors of the mitochondrial electron transport chain (HG+TTFA and HG+CCCP). HAEC were cultured and incubated for 45 mins with CM-H₂DCFDA at 37C.
Fluorescence was read using a Wallac fluorescent plate reader workstation. TTFA and CCCP significantly blocked mitochondrial ROS production, p<0.001 by ANOVA. Samples were analyzed in quadruplicate.
Online suppl Figure 1

IL8 mRNA (nanograms)

NG  L-Glu  HG

*