

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.¹ 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 β_1 integrin on the HAEC surface, suggesting that both activate the $\alpha_5\beta_1$ integrin complex on the endothelial surface. The $\alpha_5\beta_1$ 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- κ 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 $\alpha_5\beta_1$ integrin complex on HAECs. (*Circ Res.* 2003;92:371-377.)

Key Words: interleukin-8 ■ diabetes ■ endothelium ■ AP-1 ■ carbohydrate response element

Monocytes are the primary inflammatory cells that are localized to human atherosclerotic plaques.^{2,3} Studies have shown the importance of monocyte recruitment to endothelium for atherosclerosis development.⁴⁻⁶ During inflammation, monocytes are recruited to sites of endothelial cell injury and roll along the vascular endothelium, where they become activated by soluble or surface-bound chemokines. The monocytes adhere firmly to the endothelium and transmigrate through the endothelial cell (EC) monolayer.⁷⁻⁹ E-, L-, and P-selectin are involved in mediating monocyte rolling along the endothelium, and β_1 and β_2 integrins are involved in mediating firm adhesion. Vascular cell adhesion molecule-1 (VCAM-1) and an alternatively spliced form of fibronectin, connecting segment-1 (CS-1), are also involved in monocyte rolling and adhesion.^{1,10,11}

Interleukin-8 (IL-8) is a chemokine produced by endothelial cells in response to inflammatory stimuli. IL-8 is a member of the CXC class of chemokines and is chemotactic for neutrophils. However, Gerszten et al¹² found that IL-8 mediates monocyte recruitment and firm adhesion to the endothelium. Ley and colleagues¹³ have identified the chemokine KC (the mouse homolog of IL-8) as being the

principal chemokine that triggers monocyte arrest in carotid arteries with early atherosclerotic lesions. Both mildly oxidized LDL and TNF- α can induce IL-8 mRNA in endothelial cells.¹⁴ Bone marrow transplantation from mice lacking CXCR2 into LDL receptor-deficient mice caused reduced atherosclerosis development in the recipient mice,⁶ indicating that IL-8 plays an important role in macrophage accumulation in atherosclerotic lesions.

Diabetes is an independent risk factor for the development of atherosclerosis. Atherosclerosis is a major complication of patients with type 2 diabetes.¹⁵⁻²¹ We have previously shown that glucose increases monocyte adhesion to endothelial cells in vitro through increasing deposition of CS-1 fibronectin on the EC surface.¹ The mechanisms by which glucose increases monocyte adhesion to EC are not fully understood, and understanding these mechanisms will prove important for future therapeutic prevention of cardiovascular disease in diabetes.

In the present study, we examined mechanisms by which glucose activates endothelial cells to trigger monocyte:endothelial interactions. We found that glucose caused significant production of IL-8 in human endothelial cells. This induction

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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*-chlorophenylhydrazine and thenoyltrifluoroacetone were purchased from Sigma. Calcein 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 antibody²² directed against the active conformation of β_1 integrin was purchased from Pharmingen. NF- κ B (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.¹ HAECs were cultured for 7 days in Medium 199 containing 20% heat-inactivated FBS, 20 μ g/mL ECGS, and 90 μ g/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.¹ For studies using chemical uncouplers of mitochondrial function, HAECs were cultured as described above and treated for 7 days with 0.5 μ mol/L carbonyl cyanide *m*-chlorophenylhydrazine or 10 μ mol/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 scraper and collected in M199 supplemented with 20% heat-inactivated FBS, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 30 μ g/mL of ECGS. PAECs were plated into 0.1% gelatinized flasks and used from passages 3 to 6.

Human Monocyte Adhesion Assay

Methods describing the monocyte adhesion assay have been published previously¹; however, detailed methods can be found online in the expanded Materials and Methods section in the data supplement available at <http://www.circresaha.org>.

Quantitative PCR for IL-8

Total cellular RNA was obtained from HAECs using Trizol. Reverse transcription of 2 μ g of total RNA was performed in a total volume of 25 μ L. DNase-treated total RNA was reverse transcribed using random hexamers and Superscript II according to the manufacturer's protocol. For quantitative measurements of IL-8 mRNA, 2 μ L of cDNA from each experimental group were utilized. In this reaction, forward and reverse primers for human IL-8 and a Taqman internal 5'-FAM-labeled probe oligonucleotide for human IL-8 were used in a PCR reaction. The PCR reaction included 40 cycles of amplification at 94°C for 30 seconds, and 60°C for 1 minute followed by 1 extension cycle of 10 minutes at 72°C. Multiplexed in the same reaction mix were forward and reverse primers and a Taqman 5'-VIC-labeled probe oligonucleotide for human GAPDH. Quanti-

tative PCR was performed using a BioRad icycler PCR instrument equipped with a real-time camera detection module. Nanograms of IL-8 mRNA were calculated by the standard curve method using a pool of HAEC cDNA and normalizing to GAPDH levels obtained for each sample.

IL-8 ELISA

HAECs were cultured in 60 mm or 100 mm dishes as indicated, and supernatants were collected. ELISA for IL-8 in supernatants was performed using human IL-8 ELISA kits according to the manufacturer's instructions. In general, supernatants were diluted 1:50 for ELISA and quantitated using a standard curve of recombinant human IL-8. IL-8 secretion into media was represented as pg released/mg total cell protein to normalize for cell number differences in each experimental condition.

Cell Surface Integrin ELISA

Detailed methods describing the β_1 integrin cell surface ELISA can be found in the online data supplement.

Isolation of Mouse Aortic Endothelial Cells

Aortic endothelial cells from C57BL/6J and db/db mice (MAECs) were harvested from mouse aorta under sterile conditions. The aorta was excised, all periadventitial fat was removed, and the aortic pieces were placed onto Matrigel in DMEM+15% HI-FBS. After 3 days, the explants were removed, and the endothelial cells allowed to grow to confluence. Cells were passaged using dispase, and cultured for 2 days in DMEM +15% HI-FBS containing D-valine to rid of possible fibroblast contamination. After 2 days, the cells were returned to medium without D-valine. EC cultures are tested for purity at passage 2 using di-acetylated LDL and were used in experiments from passages 3 to 6.

Mouse 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.^{23,24} WEHI were cultured in DMEM +10% heat-inactivated FBS. For adhesion assays, MAECs from C57BL/6J and db/db mice were cultured in 48-well plates. WEHI cells are 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 \times 10 eyepiece grid was counted using fluorescent microscopy.

Promoter Studies

The human IL-8 promoter-reporter construct contained -1481 to +44 bp of the human IL-8 promoter. Plasmid constructs of the human IL-8 promoter containing a mutated NF- κ B site or an mutated AP-1 site were generated as described previously.¹⁴ The NF- κ B element was mutated from TGAATTTCT to TGGAAATTTaaa. The AP-1 element was mutated from TGACTCA to TGACTgt. For transient transfections, PAECs were grown in 5.5 mmol/L (NG) or 25 mmol/L (HG) glucose for 7 days on gelatin-coated plates as described above. PAECs 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.²⁵ Thus, we utilized PAECs in the transfection studies. PAECs were transfected in 12-well plates with 2 μ g 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.

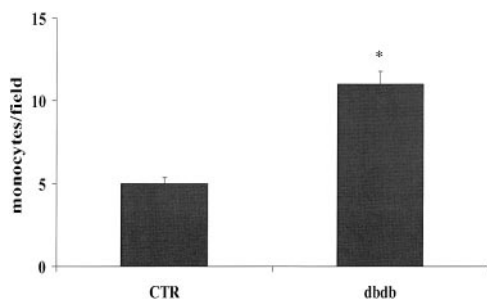


Figure 1. Monocyte adhesion is increased to endothelial cells from diabetic mice. Endothelial cells were isolated from aorta of C57BL/6J (CTR) and diabetic (db/db) mice. Cells were used from passages 3 to 6. Adhesion assays using WEHI cells, a mouse monocyte cell line, were performed as described in Materials and Methods. *Significantly higher than CTR, $P < 0.001$. Data represent the mean \pm SE of 5 experiments.

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay

HAECs were cultured in NG and HG as described above. Cells were harvested by scraping with a cell scraper. Nuclear proteins were prepared using the CellLytic NuCLEAR extraction kit from Sigma. Nuclear proteins were quantitated using a BioRad DC protein assay kit.

Electrophoretic mobility shift assays for NF- κ B and the carbohydrate (glucose) response element (CHO-RE) were performed as described¹⁴ using 5 μ g nuclear extract. The sense strand of the double-stranded NF- κ B oligonucleotide probe is 5'-AGTTGAGGGGACTTCCAGGC-3', and the sense strand of the double stranded AP-1 oligonucleotide probe is 5'-CGCTTGATGAGTCAGCCGGAA-3'. The sense strand of the double stranded CHO-RE is 5'-GCCAGTCTCACGTGGTGCC-3'. This oligonucleotide sequence for CHO-RE has been used successfully by Towle and colleagues to examine regulation of hepatic genes by glucose.^{26,27}

Statistical Analyses

Data for all experiments were analyzed by ANOVA and Fisher's protected least significant difference test using the Statview 6.0 software program. Data are represented as the mean \pm SE of 5 different experiments unless otherwise noted in the figure legends.

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.²⁸ At 6 to 12 weeks of age, these mice are used as a model of type 2 diabetes.²⁸ 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.¹ 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

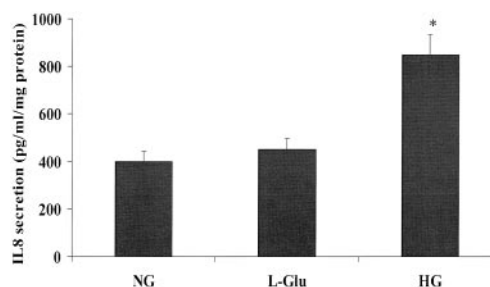


Figure 2. IL-8 production is induced in HAECs by glucose. HAECs were incubated in 5.5 mmol/L (NG), 25 mmol/L glucose (HG) for 7 days, or 25 mmol/L L-glucose for 7 days (L-Glu). Media were collected, and secreted IL-8 was measured using ELISA for human IL-8. * $P < 0.009$ vs NG by ANOVA. Data represent the mean \pm SE of 5 experiments.

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.¹ CS-1 is an adhesion molecule that can bind to the $\alpha_5\beta_1$ integrin complex on the EC surface.¹⁰ To determine the effects of glucose on β_1 integrin activation, we used the monoclonal antibody HUTS-21.²² The HUTS-21 antibody recognizes only

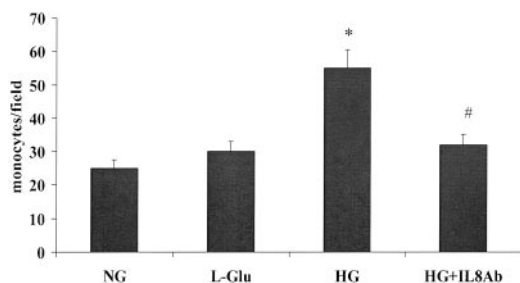


Figure 3. Neutralizing antibody to IL-8 blocks monocyte adhesion. HAECs were cultured for 7 days in 5.5 mmol/L (NG), 25 mmol/L (HG) glucose, or 25 mmol/L L-glucose (L-Glu). Neutralizing antibody to IL-8 was incubated with HG cells (HG+IL8Ab; 20 μ g/mL) for 2 hours before a monocyte adhesion assay. *Adhesion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

the activated form of β_1 integrin, so this antibody can be used as a specific measure of β_1 integrin complex activation. Glucose and IL-8 both significantly activated β_1 integrin on the EC surface to a similar extent (Figure 4). These data suggest that IL-8 triggers monocyte arrest through activation of β_1 integrin. Combined, these studies illustrate the key role of IL-8 in mediating monocyte:endothelial interactions.

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, pGL₂Basic (Promega) that contained -1481 to $+44$ bp of the human IL-8 promoter. Analysis of the human IL-8 promoter using

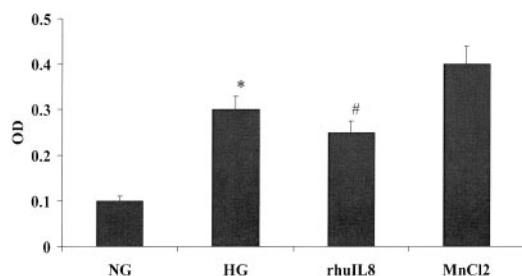


Figure 4. Glucose and IL-8 activate β_1 integrin on the endothelial surface. HAECs were cultured for 7 days in 5.5 mmol/L (NG) or 25 mmol/L (HG) glucose or incubated for 4 hours with 5 ng/mL recombinant human IL-8 (rhuIL8). Cell-surface ELISA for activated β_1 integrin was performed as described in Materials 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 β_1 integrin activation. Data represent the mean \pm SE of 3 experiments.

Relevant Transcription Factor Binding Sites* Within the Human IL-8 Promoter (Bases -1481 to $+44$)

Transcription Factor	Location
AP-1	Between -112 and -130 †
CREB	-1309
NF- κ B	Between -112 and -130 †
Putative E-boxes/bHLH (CHO-RE)‡	-97 and -102
	-476 and -481
	-686 and -691
	-743 and -748
	-1089 and -1094
	-1156 and -1161
	-1183 and -1188
	-1226 and -1231
	-1385 and -1390

*Identified using MacVector 7.0 software unless otherwise noted.

†Based on data of Abe et al.³⁰ and Mahe et al.³¹

‡Nine possible binding sites (paired; located within 5 bases of each other). Pairing based upon data of Shih et al.³⁴

MacVector software (GCG, Inc) revealed binding sites for NF- κ B 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- κ B and AP-1 promoter elements in glucose-mediated activation of IL-8. Using a human IL-8 promoter construct that contained a mutated NF- κ B 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- κ B binding to endothelial nuclear extracts (NF- κ B 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

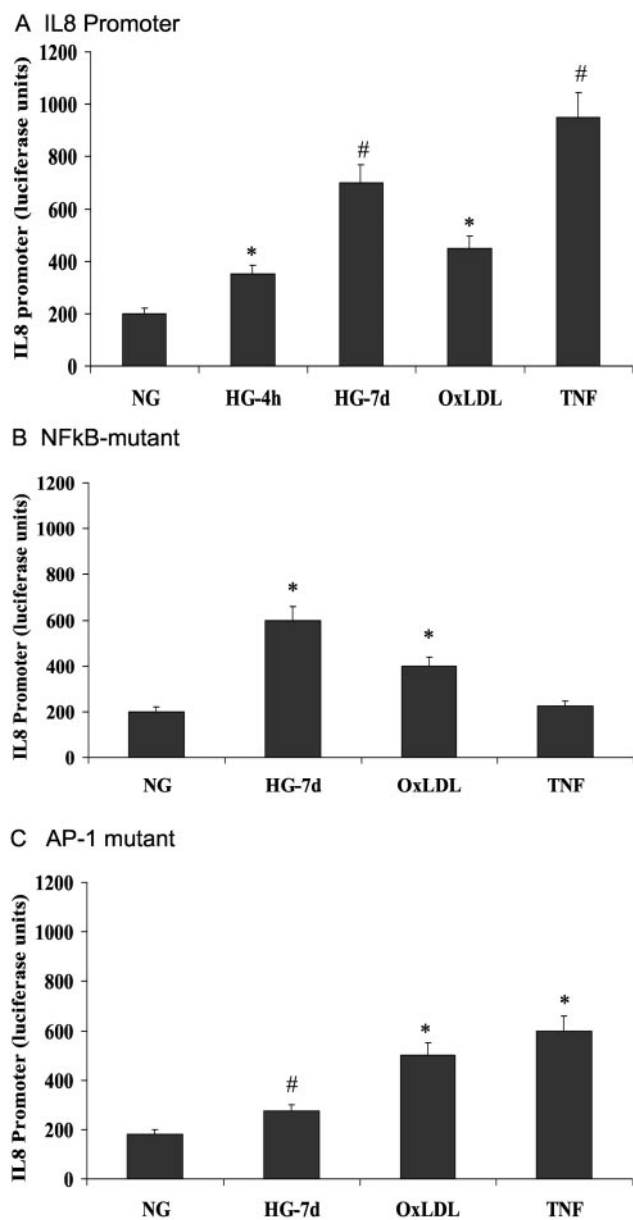


Figure 5. Glucose activates the human IL-8 promoter through activation of AP-1. PAECs were cultured in 5.5 mmol/L (NG) or 25 mmol/L glucose for 4 hours (HG-4h) or 7 days (HG-7d). A, PAECs were transfected with a plasmid containing -1481 to +44 bp of the human IL-8 promoter for 48 hours before measurement 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 μ g/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 NF- κ B 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 NF- κ B. *Significantly higher than NG by ANOVA, $P < 0.001$. Data represent the mean \pm 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

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 μ -chlorophenylhydrazone (CCCP) disrupts the proton gradient through uncoupling of mitochondrial oxidative phosphorylation.³² 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

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.¹² 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.³³ The studies of Gerszten and colleagues¹² illustrated a new role for IL-8 in mediating monocyte rolling, and the recent elegant studies of Ley and colleagues¹³ 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 Berliner¹⁴ have recently shown that IL-8 is a mediator of oxidized phospholipid activation of monocyte

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 \pm SE of 5 experiments performed in triplicate.

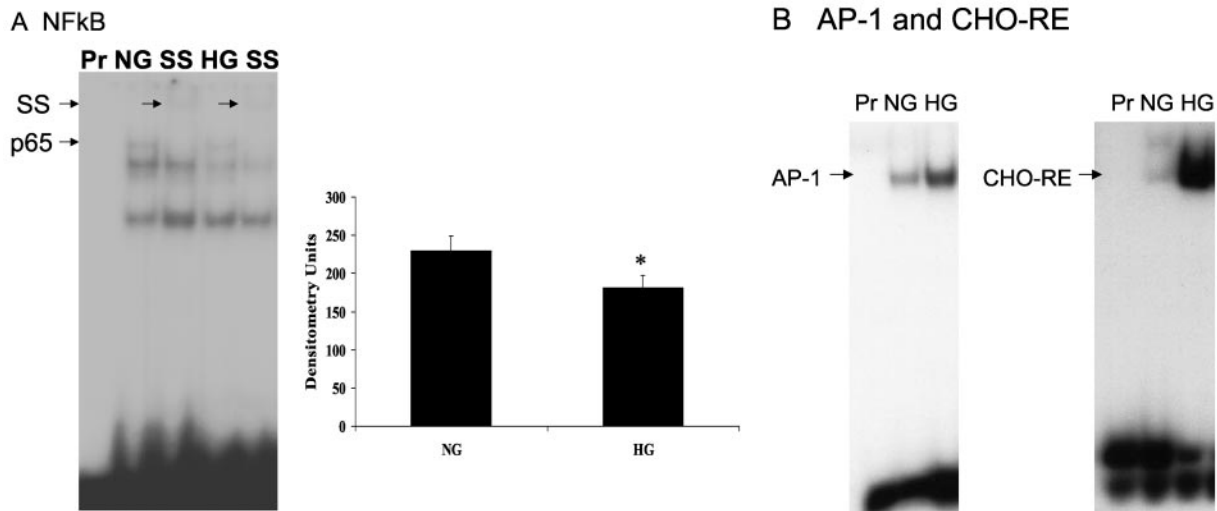


Figure 6. AP-1 and CHO-RE elements are important in glucose-mediated activation of IL-8 in endothelial cells. A, EMSA for NF- κ B was performed in nuclear extracts from endothelial cells cultured in 5.5 mmol/L glucose (NG) or 7 days in 25 mmol/L glucose (HG) as described in Materials and Methods. Bands 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).

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 β_1 integrin (Figure 4). The $\alpha_5\beta_1$ endothelial integrin complex binds to CS-1 fibronectin, and CS-1 is a counter-receptor for VLA-4 on monocytes.¹⁰ We have previously shown that glucose upregulates CS-1 fibronectin deposition on the apical surface of aortic endothelial cells.¹ IL-8 may contribute to adhesive events through activation of β_1 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

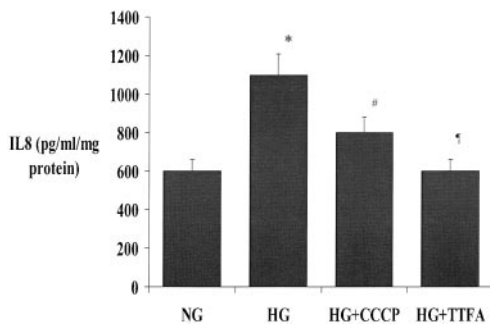


Figure 7. Mitochondrial ROS production is linked to IL-8 secretion. HAECs were cultured for 7 days in 5.5 mmol/L glucose (NG) or 25 mmol/L glucose (HG) in the presence of inhibitors of the mitochondrial electron transport chain (HG+TTFA and HG+CCCP). IL-8 secretion into HAECs media was measured by ELISA 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.

in glucose. These data indicate the activation of secondary transcriptional element(s). As shown in Figures 5 and 6, NF- κ B elements appeared to be responsible for only a small part of IL-8 promoter activation in response to glucose. Although NF- κ B appeared to play some role in IL-8 promoter activation, ECs cultured in glucose showed less binding to a labeled NF- κ B consensus sequence. These data suggest that glucose could be downregulating a repressor of the NF- κ B response element. Or, it could be that NF- κ B 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- κ B, 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

further investigate the role of mitochondrial function in mediating monocyte:endothelial interactions.

In summary, we have found that endothelial cells cultured under long-term glucose conditions have increased production of ROS and IL-8. IL-8 mediates monocyte:endothelial interactions, and inhibition of IL-8 blocks glucose-mediated adhesion. We found that glucose regulates IL-8 production at the level of transcription, and that this effect is mediated, at least in part, by AP-1 and CHO-RE elements located within the IL-8 promoter.

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