Modulation of PPARα Expression and Inflammatory Interleukin-6 Production by Chronic Glucose Increases Monocyte/Endothelial Adhesion

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Objective—We have previously reported increased monocyte adhesion to human aortic endothelial cells (HAECs) cultured in 25 mmol/L glucose (HG) compared with normal glucose (NG) (5.5 mmol/L). In this study, we explored mechanisms that contribute to increased monocyte adhesion by elevated glucose.

Methods and Results—We found that HAECs cultured in HG have increased production of the chemokine interleukin-6 (IL-6). We examined whether IL-6 directly modulated monocyte adhesion to EC. Inhibition of IL-6 using a neutralizing antibody significantly reduced glucose-mediated monocyte adhesion by 50%, and addition of IL-6 directly to human EC stimulated monocyte adhesion. PPARα has been reported to negatively regulate expression of IL-6 in vascular cells, so we examined PPARα-associated signaling in EC. A known PPARα agonist, Wy14,643, prevented glucose-mediated IL-6 production by EC and reduced glucose-mediated monocyte adhesion by 40%. HG-cultured HAEC had a 50% reduction in expression of PPARα compared with control EC. Primary aortic EC isolated from PPARα knockout (KO) mice showed increased monocyte adhesion compared with EC isolated from control mice. PPARα KO EC also had increased production of IL-6. Finally, we measured IL-6 levels in diabetic db/db mice and found significant 6-fold elevations in IL-6 levels in db/db EC.

Conclusions—These data indicate that IL-6 production is increased in diabetes and contributes to early vascular inflammatory changes. PPARα protects EC from glucose-mediated monocyte adhesion, in part through regulation of IL-6 production. (Arterioscler Thromb Vasc Biol. 2004;24:851-857.)

Key Words: endothelium ▪ monocytes ▪ PPARα ▪ atherosclerosis ▪ interleukin-6
tion.\textsuperscript{22,23} PPARα regulates expression of COX-2, IL-1β, IFNγ, TNFα, IL-6, IL-8, and MCP-1, mostly to downregulate expression of these pro-inflammatory molecules,\textsuperscript{18,24,25} except in a few cases.\textsuperscript{26} PPARα inhibits expression of IL-6 via negative regulation of nuclear factor-kappa B and AP-1.\textsuperscript{19,27} The PPARα agonist, Wy14,643, reduces IL-6 expression in vivo in C57BL/6J mice.\textsuperscript{28} Furthermore, PPARα KO mice have increased production of IL-6 mRNA in aorta in response to a bacterial lipopolysaccharide (LPS) challenge.\textsuperscript{19} PPARα activation has been shown to reduce atherosclerosis in mice, although the role of PPARα in mediating atherosclerosis remains unclear.\textsuperscript{29,30} Recent studies by Semenkovich et al reported that deficiency of PPARα in low-density lipoprotein receptor-deficient mice inhibited development of atherosclerosis.\textsuperscript{26}

In this study, we examined additional mechanisms for glucose regulation of monocyte adhesion to EC. We examined glucose-mediated regulation of PPARα expression and its impact on events related to monocyte adhesion. We also examined the role of IL-6 in mediating monocyte adhesion to EC in the setting of diabetes.

**Methods**

**Reagents**

Tissue culture media and reagents were purchased from Invitrogen. Fetal bovine serum was obtained from Hyclone. Calcein-AM was purchased from Molecular Probes. Human and mouse IL-6 ELISA kits were obtained from R&D Systems. Wy 14 643 was purchased from Biomol. Human IL-6 neutralizing antibody, human IL-8 neutralizing antibody, recombinant human IL-6, and recombinant human IL-8 were all purchased from R&D Systems. WEHI7/24 cells were a kind gift from Dr Judith A. Berliner (UCLA). Pioglitazone (Takeda Pharmaceuticals) was a kind gift from Dr Milagros Huerta (University of Virginia). Antibody to human PPARα was purchased from Affinity BioReagents, and antibody to histone H1 was purchased from Santa-Cruz Biotechnology (sc-8030).

**Human EC Culture**

Human aortic endothelial cells (HAEC) were obtained from aortic rings of explanted donor hearts. Use of HAEC was approved by the University of Virginia Institutional Review Board, and all procedures were performed in accordance with University Institutional Review Board guidelines. Briefly, HAEC were cultured for 7 days in medium 199 containing 20% heat-inactivated FBS, 20 μg/mL endothelial cell growth supplement (ECGS), and 90 μg/mL heparin in the absence (NG) and presence of 25 mmol/L D-glucose (HG) or 25 mmol/L L-glucose (as an osmotic control). The 7-day, 25-mmol/L HG incubation condition was chosen because monocyte adhesion to EC was maximal at this concentration of glucose and time-point of incubation.\textsuperscript{31}

**Mouse Aortic EC Isolation**

Aortic EC from PPARα KO mice\textsuperscript{23} (Jackson Laboratories stock 003580) on a 129S4/SvJae background, PPARα control 129S1/SvImJ mice (Jackson Laboratories stock 002448), diabetic B6.Cg-\textsuperscript{m10Lepr}\textsuperscript{db/db} mice on a C57BL/6J background (Jackson Laboratories stock 000664) were harvested from mouse aorta after modifications of the method described previously.\textsuperscript{32} These methods were approved and performed under the guidelines established by the University of Virginia Animal Care and Use Committee. Briefly, the aorta is excised, all periaortic fat is removed under a magnifying scope, and the aortic pieces are placed onto Matrigel in DMEM plus 15% HI-FBS. The EC grow out from these aortic explants. After 3 days, the explants are removed, and the EC are grown to confluence as described.\textsuperscript{33} EC are routinely used from passages 2 to 4. For the studies using diabetic db/db EC, cells were isolated as described and cultured for 1 passage in DMEM containing 15% heat-inactivated FBS, 60 μg/mL ECGS, and 100 μg/mL heparin, and 5.5 mmol/L glucose before performing assays. We have previously reported that diabetic db/db EC retain memory of their diabetic milieu during short-term passage in culture.\textsuperscript{33}

**Monocyte Adhesion Assays**

Monocyte adhesion assays using human cells were performed as described previously.\textsuperscript{2} Briefly, NG- and HG-cultured HAEC were cultured to confluence as described into 48-well plates. HAEC were rinsed with 1% M199. Human primary monocytes were isolated from healthy normal volunteers using a modification of the Recalde method\textsuperscript{34} and labeled with Calcein AM (Molecular Probes) for 10 minutes at 37°C. Labeled human primary monocytes (50 000/well) were added to HAEC monolayers and incubated for 30 minutes at 37°C. Unbound monocytes were rinsed away, cells were fixed in 1% glutaraldehyde, and bound labeled monocytes were counted within a 10×10 grid using epifluorescence microscopy. Cells were incubated at 37°C with 10 U/mL recombinant human TNFα for 4 hours as a control to show maximal monocyte adhesion in our assays. For subsets of studies, NG- and HG-cultured HAEC were incubated at 37°C with 100 μmol/L Wy14,643 or 5 μmol/L pioglitazone for 4 hours before adhesion assays. For IL-6 studies, HAEC were incubated at 37°C with recombinant human IL-6 (5 ng/mL) for 4 hours, IL-6 neutralizing antibody (0.1 μg/mL) for 4 hours, or an irrelevant antibody (α-mouse IgG; 0.1 μg/mL) for 4 hours before performing a monocyte adhesion assay. For IL-8 studies, HAEC were incubated at 37°C with either recombinant human IL-8 (5 ng/mL) for 4 hours or IL-8 neutralizing antibody (20 μg/mL for 2 hours) before performing a monocyte adhesion assay.

**Mouse Assays**

Our laboratory has recently developed a monocyte adhesion assay that uses primary mouse aortic EC and WEHI 78/24 mouse monocytes.\textsuperscript{13} WEHI 78/24 is a mouse monocyte cell line that has been well characterized.\textsuperscript{35} WEHI are cultured in 10% heat-inactivated FBS in DMEM containing 4.5 g/L glucose. WEHI are labeled with Calcein AM (Molecular Probes) immediately before experiments according to manufacturer’s instructions. Mouse aortic EC are incubated with 35 000 fluorescent WEHI cells/well for 30 minutes at 37°C. Nonadherent cells are rinsed and the cells fixed with 1% glutaraldehyde. The number of attached monocytes present within a 10×10 grid is counted using epifluorescence microscopy.

**Enzyme-Linked Immunosorbent Assay for Human and Mouse IL-6**

Supernatants from cultured EC were collected, aliquoted to prevent repetitive thawing, and stored at −20°C. Enzyme-linked immunosorbent assay (ELISA) for human and mouse IL-6 in supernatants was performed using ELISA kits according to the manufacturer’s instructions. Supernatants were used undiluted in quadruplicate wells/sample. IL-6 levels in supernatant were determined using a standard curve. For normalization purposes, EC lysates were harvested using sodium dodecyl sulfate (SDS) lysis buffer containing phenylmethylene sulfonyl fluoride and protease inhibitor cocktail (Sigma) and total cell protein was measured using a BioRad protein assay. IL-6 secretion into media was represented as picograms released into media/ng total cell protein to normalize for possible cell number differences under each experimental condition.

**Nuclear Protein Extraction and Immunoblotting of PPARα**

HAEC were cultured in NG and HG as described. After rinsing the monolayer of cells twice with PBS, cells were harvested using a cell scraper and lysed using cell lysis buffer (10 mmol/L HEPES pH 7.9, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.1 mmol/L DTT, and 10 μL of Sigma protease inhibitor cocktail). Cells were incubated on ice for 15 minutes. Then 10% Igepal CA-630 was added to a final
Glucose Increases IL-6 Production by HAEC

The concentration of 0.6%, and cells were vortexed vigorously and centrifuged at 10,000g for 3 minutes. Cell pellets were resuspended in extraction buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl2, 0.42 mM NaCl, 0.2 mM EDTA, 25% glycerol with Sigma protease inhibitor cocktail, and 0.1 mM DTT) and agitated on a vortex mixer for 30 minutes. The nuclear extracts were separated by centrifugation at 20,000g for 5 minutes and aliquots were stored in −70°C. Protein was quantitated using a BioRad protein assay. SDS-PAGE of 25 μg nuclear protein was performed using 12% NuPAGE gels (Invitrogen). An antibody to human PPARα was incubated with the blots overnight at 4°C at a 1:1000 dilution. Blots were also incubated with antibody to histone H1 at a 1:500 dilution. Blots were normalized to histone H1 expression using densitometry (ZeroD Scan software; Stratagene).

Statistical Analyses

Data for all experiments were analyzed by ANOVA and Fisher protected least significant difference test using the Statview 6.0 software program. Data are represented as the mean±SE of 5 experiments.

Results

Glucose Increases IL-6 Production by HAEC

We have previously reported increased production of IL-8 in HAEC mediated by chronic elevated glucose. In the current study, we found that HAEC cultured chronically for 7 days have increased production of IL-6 protein (Figure 1). HAEC cultured in 25 mmol/L L-glucose for 7 days as an osmotic control showed no change in IL-6 production.

IL-6 Directly Stimulates Monocyte Adhesion to EC

We have previously reported that chronic elevated glucose increases monocyte adhesion to HAEC. These data are illustrated in Figure 2, in which HG-cultured HAEC (HG) have a 2-fold increase in monocyte adhesion compared with EC cultured in 5.5 mM glucose (NG). There was no effect of L-glucose on monocyte adhesion to EC. TNFα is used as a positive control to indicate maximal monocyte adhesion in the assay.

Several studies have suggested that IL-6 may mediate monocyte recruitment to activated endothelium. However, whether IL-6 directly can stimulate monocyte/EC adhesion is unclear. We tested this hypothesis and found that addition of recombinant human IL-6 to HAEC directly stimulated monocyte adhesion by 50% (NG+IL6; Figure 2). Furthermore, inhibition of endothelial IL-6 action through use of an IL-6 neutralizing antibody significantly reduced glucose-mediated monocyte adhesion to HAEC by 50% (HG versus HG+IL6Ab; Figure 2), suggesting that IL-6 action contributes significantly to glucose-mediated monocyte adhesion to EC.

We have previously reported that IL-8 plays a major role in glucose-stimulated monocyte adhesion. In this study, we directly compared the contributions of IL-6 and IL-8 in glucose-mediated monocyte adhesion. As shown in Figure 2, addition of recombinant human IL-8 to HAEC directly stimulated monocyte/endothelial interactions (NG versus NG+IL8). In addition, inhibition of endothelial IL-8 action through use of an IL-8 neutralizing antibody significantly reduced glucose-mediated monocyte adhesion by ≈50% (HG versus HG+IL8Ab; Figure 2). Thus, IL-6 is an important contributor, yet not the sole contributor, to glucose-stimulated monocyte adhesion. IL-6 and IL-8 contribute almost equally to regulate glucose-mediated monocyte adhesion.
Glucose Decreases PPARα Expression in EC

PPARα expression may negatively regulate IL-6 production in an anti-inflammatory manner. In Figure 1, we found an increase in IL-6 production in HG-cultured HAEC, so one explanation for an increase in IL-6 production could be a decrease in PPARα expression or function. We tested whether PPARα expression in HAEC was reduced by chronic elevated glucose. We found that PPARα protein levels were reduced by \( \approx 50\% \) in HAEC cultured in chronic glucose (HG) \( (P<0.001; \text{Figure 3}) \).

We also found that the known PPARα agonist, Wy14,643 prevented complete downregulation of PPARα protein in response to glucose in EC (HG+Wy; Figure 3). Wy14,643 also significantly increased PPARα protein levels in normal HAEC (NG+Wy; Figure 3). We have recently found that Wy14,643 increases PPARα mRNA expression in EC (data not shown). Regulation of PPARα mRNA transcription by Wy14,643 has been reported by Sterchele and Mukherjee. These data suggest that Wy14,643 acts as an agonist and also regulates PPARα expression, probably through transcriptional processes.

The PPARα agonist Wy14,643 completely blocked glucose-mediated IL-6 production in HAEC (Figure 4A). Wy14,643 also significantly reduced glucose-mediated monocyte adhesion to HAEC by \( \approx 40\% \) (Figure 4B). TNFα was used as a positive control to show maximal adhesion in this assay. We also examined the effects of the PPARγ agonist pioglitazone on monocyte adhesion. We found no reduction in glucose-mediated monocyte adhesion using 5 \( \mu \)mol/L pioglitazone for 4 hours (Figure 4B). These data indicate that glucose increases monocyte adhesion in part through downregulation of PPARα expression with a resulting increase in production of IL-6. These data also suggest that IL-6 is responsible for \( \approx 50\% \) of glucose-mediated monocyte adhesion.

PPARα-Deficient Mice Have Increased Monocyte/Endothelial Interactions and Increased IL-6 Expression

To further examine the role of endothelial PPARα in glucose-mediated monocyte adhesion, we used primary aortic EC isolated from PPARα KO mice on a B6,129 S4 background. EC usually do not bind monocytes unless they are activated.
As shown in Figure 5, monocyte adhesion was significantly increased 2-fold in basal, unstimulated EC isolated from PPARα KO mice compared with EC from control mice (control strain 129S1/sVImJ; \( P<0.009 \)). Furthermore, we measured IL-6 production in control and PPARα KO aortic EC. We found a dramatic 4-fold increase in IL-6 production in aortic EC of PPARα KO mice compared with control mice (Figure 5) and a 2-fold increase in IL-6 mRNA levels in PPARα KO mice (data not shown). The Wy compound had no effect on monocyte adhesion or IL-6 production in PPARα KO mice (data not shown). This is consistent with earlier data from Gonzalez et al who found no effects of Wy14,643 on cellular parameters in the PPARα KO mice.\(^{38-41}\) We also found that exposing PPARα KO EC to elevated glucose (25 mmol/L for 7 days) increased monocyte adhesion but had no effect on IL-6 production (data not shown). This is important in that we have shown regulation of monocyte adhesion by additional chemokines that are not regulated by PPARα, including IL-8.\(^{31}\) However, IL-6 production appears to be primarily regulated by PPARα. Taken together, our data using PPARα KO mice indicate that PPARα expression is important for regulation of monocyte/endothelial adhesion, and that this regulation occurs, at least in part, through modulation of endothelial IL-6 production.

**Diabetic Mice Also Show Increased Production of IL-6**

We wanted to determine that IL-6 levels were also increased in diabetic db/db mice to illustrate the importance of this chemokine pathway in diabetes in vivo. EC were freshly isolated from mouse aortas of diabetic db/db and control mice and cultured for 1 passage in 5.5 mmol/L glucose. IL-6 levels were measured in control and diabetic EC. We found a significant 6-fold increase in IL-6 production in diabetic db/db mouse EC (Figure 6). We also found that IL-6 mRNA was elevated in db/db EC (data not shown), suggesting that IL-6 is regulated at the level of mRNA abundance in diabetic db/db mouse EC. EC from db/db mice also display increased monocyte adhesion (Figure 6). We have previously reported increased monocyte adhesion to EC in diabetic db/db mice.\(^{33}\) Thus, these data indicate a significant role for IL-6 in mediating monocyte adhesion to EC in the setting of diabetes.

**Discussion**

The signaling pathways by which glucose modulates monocyte/endothelial adhesion remain unclear. This is the first study that links glucose-mediated changes in endothelial activation to downregulation of PPARα. EC cultured in chronic glucose showed decreased expression of PPARα and increased monocyte adhesion. The studies shown in Figure 5 illustrate that absence of PPARα in murine EC results in increased monocyte adhesion. These data collectively indicate the importance of PPARα as an anti-inflammatory molecule in EC for early events of atherogenesis. In the current study, we found increased production of the pro-inflammatory chemokine IL-6 by EC in response to chronic elevated glucose. IL-6 production by EC was blocked by a PPARα agonist Wy14,643. These novel results indicate that glucose-stimulated production of IL-6 in endothelium in diabetes is mediated through inhibition of PPARα expression and/or action.

In the present study we focused on PPARα, because we have found that expression of PPARγ is low in human aortic EC.\(^{42}\) Furthermore, levels of PPARγ in human EC were not increased in response to glucose (data not shown). We found no effect of pioglitazone, a PPARγ agonist, on reducing glucose-mediated monocyte adhesion in HAEC treated for 4 hours at 37°C with 5 μmol/L pioglitazone (Figure 4). However, longer pretreatment of HAEC for 24 hours with 5 μmol/L pioglitazone slightly reduced glucose-mediated monocyte adhesion to HAEC, although the trend was not significant (data not shown). This would be consistent with another study that found that pioglitazone (20 μmol/L for 48 hours) reduced monocyte/EC interactions in response to elevated shear stress.\(^{43}\) However, other studies have not examined the role of pioglitazone in reducing glucose-stimulated monocyte adhesion. Because PPARγ levels in HAEC appear not to be regulated by glucose, and because there is a minimal effect of pioglitazone on monocyte...
adhesion in our case, taken together, our results indicate that PPARα regulation is important for glucose-mediated endothelial activation. However, detailed studies of the interaction between glucose and PPARγ in EC are needed and are underway in the laboratory.

PPARα may play a pro-inflammatory or anti-inflammatory role in the vessel wall depending on the state of oxidative stress in vascular cells. On the pro-inflammatory side, we have recently found chronic exposure of EC to glucose dramatically increases levels of reactive oxygen species in the cell. Previous work by our colleagues illustrated that PPARα stimulated MCP-1 and IL-8 synthesis in response to oxidized phospholipids and minimally modified low-density lipoprotein. Interestingly, PPARα KO mice on the apoE KO background were shown to have less atherosclerosis than control apoE KO mice. This study suggests a pro-inflammatory role for PPARα in mediating atherosclerosis. The authors suggested that the reduction of atherosclerosis in the absence of PPARα was caused by increased insulin sensitivity and enhanced glucose control. However, these investigators did not examine early events contributing to atherosclerosis that are relevant to the current study, such as monocyte/EC interactions and IL-6 production. Nevertheless, collectively, the data from these studies suggest a pro-inflammatory role for PPARα. However, many more groups have shown that PPARα is anti-inflammatory and that activation of PPARα by fibrate agonists reduces atherosclerosis development in mice. Cumard et al found that feeding C57BL/6j mice the PPAR agonist Wy14,643 reduced T cell and monocyte activation, which are key early events in formation of atherosclerotic plaques. Duez et al found that fenofibrate reduced aortic cholesterol content in apoE-deficient mice but did not reduce lesion area in the aorta. However, when these apoE KO mice were crossed with human apoAI transgenic mouse, fenofibrate dramatically reduced atherosclerotic lesion development. Mechanisms for the reduced atherosclerosis in response to PPARα activation include inhibition of T cell and monocyte activation, reduction of endothelial MCP-1 expression, and activation of apoAI. Although in most cases it is believed that PPARα exerts an anti-inflammatory effect, the type of inflammation (acute versus chronic) and the cause of inflammation (hyperlipidemia, hyperglycemia, oxidation) could be of importance in determining the anti- versus pro-inflammatory nature of PPARα.

To examine the direct role of PPARα in modulating monocyte adhesion, we used EC isolated from PPARα KO mice. Gonzalez et al have shown that PPARα KO mice have a significantly greater response to inflammatory stimuli compared with control mice. In adhesion assays, we found that EC isolated from PPARα KO mice bound a greater number of monocytes than did EC from control mice (Figure 5). These data indicate that in the absence of PPARα, EC are already activated to bind monocytes; thus, the PPARα KO EC are in a pro-inflammatory state. Additional studies will be needed to identify the molecular mechanisms leading to the enhanced monocyte adhesion observed in the PPARα KO EC.

We also show for the first time to our knowledge that EC from diabetic db/db mice have a significant upregulation in IL-6 production (Figure 6). We have previously reported that monocyte adhesion to EC is increased in db/db mice. These new data on IL-6 suggest that IL-6 plays a role in mediating monocyte adhesion in diabetic db/db mice. Further studies in these mice are needed to address this question. Nevertheless, these data suggest that IL-6 may be an important contributor to early vascular inflammatory events in diabetes in vivo.

In addition to the PPARα–IL-6 signaling pathway, there are several additional pathways in vascular EC that contribute to glucose-mediated monocyte adhesion. We have previously shown regulation of IL-8 synthesis in EC by chronic elevated glucose. We have found that IL-8 is a primary regulator of monocyte adhesion to EC in response to glucose, and it accounts for ~50% of glucose-mediated monocyte adhesion (Figure 2). The regulation of IL-8 production in EC by glucose does not appear to be through PPARα. However, there is significant cross-talk between PPARα and other signaling pathways, including MAP kinases; therefore, PPARα may be indirectly involved in production of other chemokines. We have recently shown upregulation of the 12/15 lipoxxygenase pathway in diabetic db/db mice as well as in human EC cultured chronically in elevated glucose. This inflammatory pathway most certainly contributes to monocyte/EC interactions in diabetes, although the exact mechanisms remain unclear. The role that these lipoxgenase eicosanoid products play in mediating monocyte/EC interactions in diabetes is currently being studied in the laboratory.

In summary, our data indicate that glucose-mediated induction of IL-6 and subsequent acceleration of monocyte adhesion occurs through modulation of levels of endothelial PPARα. These results support further development of modulators of PPARα expression or action to reduce accelerated cardiovascular disease caused by diabetes.

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