High Glucose–Induced Expression of Proinflammatory Cytokine and Chemokine Genes in Monocytic Cells

Narkunaraja Shanmugam, Marpadga A. Reddy, Mausumee Guha, and Rama Natarajan

Monocyte activation and adhesion to the endothelium play important roles in inflammatory and cardiovascular diseases. These processes are further aggravated by hyperglycemia, leading to cardiovascular complications in diabetes. We have previously shown that high glucose (HG) treatment activates monocytes and induces the expression of tumor necrosis factor (TNF)-α via oxidant stress and nuclear factor-kB transcription factor. To determine the effects of HG on the expression of other inflammatory genes, in the present study, HG-induced gene profiling was performed in THP-1 monocytes using cytokine gene arrays containing 375 known genes. HG treatment upregulated the expression of 41 genes and downregulated 15 genes that included chemokines, cytokines, chemokines receptors, adhesion molecules, and integrins. RT-PCR analysis further confirmed that HG significantly increased the expression of monocyte chemoattractant protein-1 (MCP-1), TNF-α, β_2-integrin, interleukin-1β, and others. HG treatment increased transcription of the MCP-1 gene, MCP-1 protein levels, and adhesion of THP-1 cells to endothelial cells. HG-induced MCP-1 mRNA expression and monocyte adhesion were blocked by specific inhibitors of oxidant stress, protein kinase C, ERK1/2, and p38 mitogen-activated protein kinases. These results show for the first time that multiple inflammatory cytokines and chemokines relevant to the pathogenesis of diabetes complications are induced by HG via key signaling pathways. *Diabetes* 52:1256–1264, 2003

Although it is well known that diabetes is associated with atherosclerotic and inflammatory disease, the specific cellular and molecular mechanisms involved are not fully resolved. Hyperglycemia is considered to be a major factor (1,2) and certain key pathways, factors, and mechanisms have been implicated, including oxidant stress (3,4), advanced glycation end products (5,6), aldose reductase (7), reductive stress (8), carbonyl stress (9), and protein kinase C (PKC) activity (10). The adhesion of monocytes to the endothelium followed by transmigration into the subendothelial space is one of the key early events in the pathogenesis of atherosclerosis (11). This can be mediated by the interaction of specific adhesion molecules on vascular endothelial cells with their integrin counter receptors on monocytes. Studies have demonstrated increased leukocyte-endothelial interactions in animal models of diabetes and with monocytes from diabetic individuals (11–13). Furthermore, culture of human aortic endothelial cells (HAECs) in vitro under chronic high glucose (HG) versus normal glucose (NG) conditions showed a significant increase in the binding of monocytes that was blocked by an antibody to β_2-integrins (14,15). However, the effect of culturing monocytes under HG conditions was not evaluated. We recently demonstrated that HG treatment of THP-1 monocytes or human peripheral blood monocytes leads to increased expression of the inflammatory cytokine, tumor necrosis factor-α (TNF-α), in an oxidant stress, nuclear factor (NF)-κB, and AP-1 transcription factor–dependent manner (16). HG culture also increased basal and TNF-α–induced p38 mitogen-activated protein kinase (MAPK) activity in these monocytic cells (16). However, it is not known whether other key monocyte-activating inflammatory cytokine and chemokine genes are induced under hyperglycemic conditions or whether the monocytes exhibit increased adhesive properties under these conditions. Furthermore, very few studies have examined the signaling pathways and molecular mechanisms leading to these events. The present study is designed to examine these issues by utilizing cytokine gene arrays to evaluate differential gene expression in monocytes under HG versus NG conditions and to perform subsequent characterization and functional assays. We observed that HG conditions led to significant induction of cytokines such as TNF-α and interleukin (IL)-1β and their receptors, chemokines such as monocyte chemoattractant protein-1 (MCP-1) and interferon-γ (IFN-γ)-inducible protein-10 (IP-10), the adhesive β_2-integrin receptor, and other genes, many of which are NF-κB regulated. We also examined the role of oxidant stress, PKC, and MAPKs in HG-induced MCP-1 expression. Our data indicate for the first time that HG can induce the transcription and activation of multiple monocyte inflammatory cytokine-related genes via key signaling pathways, possibly culminating in increased monocyte activation and adhesion.

RESEARCH DESIGN AND METHODS

Materials. The inhibitors used in the study were SB202190, bis-indolymaleimide, and *N*-acetylcysteine (NAC) were purchased from Calbiochem (San Diego, CA); PD98059 from Cell Signaling (Beverly, MA); and pyrrolidine

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ELISA, enzyme-linked immunosorbent assay; HAEC, human aortic endothelial cell; HG, high glucose; IFN-γ, interferon-γ; IL, interleukin; IP-10, inducible protein-10; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MN, mannitol; NAC, *N*-acetylcysteine; NF, nuclear factor; NG, normal glucose; PDTC, pyrrolidine dithiocarbamate; PKC, protein kinase C; TNF, tumor necrosis factor; TTFA, thenoyltrifluoroacetone.

TABLE 1
PCR primers sequences and PCR conditions

	Sequences (5′–3′)	Annealing temperature (°C)	Cycles (*n*)	Product size
MCP-1				
Sense	CAAACTGAAGCTCGCACTC	59	29	659 bp
Antisense	CATTTCCACAATAATATTTTAG			
β_1-integrin				
Sense	GCCTTACATTAGCACAACACC	57	35	283 bp
Antisense	ATCTCCAGCAAAGTGAAACC			
β_2-integrin				
Sense	AAAAACATCCAGCCCATCTTC	57	35	271 bp
Antisense	ATCTGCACGCCATCACAGTC			
IP-10				
Sense	TGAAAAAGAAGGGTGAGAAGAG	59	33	413 bp
Antisense	GGAAGATGGGAAAGGTGAGG			
PECAM-1				
Sense	AGGAAAGAAGGACACAGAGAC	56	33	264 bp
Antisense	ATGGATTTAAGAACCGGCAG			
IL-1 β				
Sense	CTCTCTCACCTCTCCTACTCAC	56	33	187 bp
Antisense	ACACTGCTACTTCTTGCCCC			
TNF-α				
Sense	CCAAACGATGTTGTACCCGA	59	29	251 bp
Antisense	CAGTTGGAGGAGAGACGGTA			

dithiocarbamate (PDTC), thenoyltrifluoroacetone (TTFA), and apocynin from Sigma-Aldrich Chemicals (St. Louis, MO). Human panorama cytokine gene arrays and corresponding reagents were from Sigma-Aldrich Chemicals. ^{32}P-α-dCTP (3,000 Ci/mmol) was from New England Nuclear (Boston, MA). RT-PCR reagents were from Applied Biosystems (Foster City, CA) and QuantumRNA 18S Internal Standards from Ambion (Austin, TX). Effectene, plasmid DNA isolation kits, and RNeasy and Oligotex kits were from Qiagen (Valencia, CA). Luciferase assay system was from Promega (Madison, WI). Quantikine MCP-1 enzyme-linked immunosorbent assay (ELISA) kit, anti-human TNF-α neutralizing antibody, and normal rabbit IgG were from R&D Systems (Minneapolis, MN).

Cell culture and treatments. Human THP-1 monocytic cells were obtained from American *Type Culture* Collection and cultured in RPMI-1640 medium supplemented with 10% FCS, glutamine, HEPES, streptomycin/penicillin (100 $\mu g \cdot ml^{-1} \cdot 100$ units $\cdot ml^{-1}$), and either 5.5 mmol/l D-glucose (NG) or 15 mmol/l D-glucose (HG) in a 5% CO_2 incubator at 37°C. In some experiments, THP-1 cells were pretreated with one of the following: SB202190 (p38MAPK inhibitor, 1 μmol/l), bis-indolylmaleimide (GFX, PKC inhibitor, 0.5 μmol/l), PD98059 (ERK MAPK pathway inhibitor, 25 μmol/l), NAC (thiol antioxidant that also serves as a glutathione precursor, 100 μmol/l), PDTC (antioxidant, 100 μmol/l), apocynin (30 μmol/l) (NADPH oxidase inhibitor), TTFA (mitochondrial complex II inhibitor that can block mitochondrial superoxide production, 10 μmol/l) (17), anti-human TNF-α antibody, or control IgG (50 ng/ml). The cells were then incubated in NG or HG media for a further 24 or 72 h. Inhibitors or the corresponding vehicle were readded every 24 h.

RNA preparation. Total RNAs were prepared from NG- or HG-treated THP-1 cells by the RNeasy method and poly(A) was prepared using Oligotex kit.

HG-induced differential gene expression by cDNA array analysis. The expression profile of HG-induced genes in THP-1 cells was examined using Panorama human cytokine gene arrays. A total of 1 μg poly(A) RNA isolated from the NG- or HG-treated cells was converted to radiolabeled cDNA using [α-^{32}P]dCTP and reagents provided and then hybridized to two identical cytokine gene array membranes according to manufacturer's instructions. The hybridized membranes were scanned and specifically bound radioactive spots quantitated using phosphorimager and Image Quant software (Molecular Dynamics, Sunnyvale, CA), respectively. Relative changes in mRNA levels in two independent experiments were calculated and results expressed as fold induction over NG-treated cells after normalization to the expression of three internal controls, namely GAPDH, β-actin, and cyclophilin A.

DNA transfection and luciferase assays. THP-1 cells plated in a six-well plate (1.2×10^6 per well) were transfected with 1 μg of the indicated plasmids, p420-MCP-1-Luc (generous gift from Dr. Thomas McIntyre, University of Utah, Salt Lake City, UT), the control pCR-Luc plasmid (Invitrogen), or the pCMV-mIκB plasmid (18) (generous gift from Dr. E. Zandi, University of Southern California, Los Angeles, CA) using Effectene transfection reagent. The transfected cells were cultured for 72 h in either NG or HG medium, washed with PBS, lysed with 100 μl of lysis buffer, and stored overnight at –70°C. Samples were thawed, brought to room temperature, and 20 μl of each lysate used to analyze luciferase activity.

Relative RT-PCR. Total RNA was isolated from THP-1 cells (2×10^6/sample), grown in NG or HG for 72 h, and 1 μg RNA used for the RT reaction using a Gene Amp RNA PCR kit. cDNA corresponding to 0.05 μg RNA was then used in multiplex PCRs containing gene-specific primers (Table 1) paired with Quantum RNA 18S Internal Standards. Multiplex PCRs were performed for 25–35 cycles in a GeneAmp 9700 machine (Applied Biosystems). PCR products were fractionated on 2.5% agarose gels, photographed using AlphaImager 2000, and quantitated with Quantity 1 software (Bio-Rad Laboratories, Hercules, CA). Results were expressed as fold stimulation over NG after normalizing with paired 18S RNA levels.

Measurement of MCP-1 levels by ELISA. Supernatants of THP-1 cells (5×10^5 cells/ml) cultured in RPMI 1640 medium containing 2% FBS for 3 days under NG or HG conditions were used to assay secreted MCP-1 levels using a specific Quantikine ELISA kit. Medium alone without cells was incubated under the same conditions and used as blank control for the ELISA.

Monocyte adhesion assays. THP-1 cells were cultured in NG or HG (15 mmol/l) for 2 days and then in serum-depleted media for 24 h. Adhesion experiments were carried out as described earlier (14). In experiments where we examined the effects of inhibitors, THP-1 cells were incubated for 24 h with HG in the presence or absence of the indicated inhibitors or the corresponding vehicles.

Data analyses. Data are expressed as mean ± SE of multiple experiments. Paired Student's *t* tests were used to compare two groups and ANOVA for multiple comparisons. The gene array experiments were performed in duplicate. At least two images from each set of arrays were collected and the analyses performed as described in RESEARCH DESIGN AND METHODS and RESULTS.

RESULTS

HG-induced expression of proinflammatory cytokine, chemokine, and related genes. We tested the hypothesis that HG can induce the transcriptional regulation of inflammatory and pathologic genes as well as their receptors via specific signaling pathways. This could result in increased monocyte activation, migration, and adhesion to the endothelium. Our recent study showed that HG can induce the transcriptional regulation of TNF-α gene in monocytes (16). However, it is highly likely that several other important and relevant genes other than TNF-α could be induced in monocytes under HG/diabetic condi-

tions. We therefore used human cytokine cDNA arrays as a tool to identify key chemokines and cytokine-related genes altered under diabetic conditions. Each gene array consists of 375 sequence-verified human cDNA clones of cytokine and immunoregulatory genes that are spotted in duplicate onto a charged nylon membrane. The array also includes nine housekeeping genes to compare relative changes in gene expression between control and test samples hybridized to two separate arrays.

Poly(A) RNA was extracted from THP-1 cells treated with NG (5.5 mmol/l) or HG (15 mmol/l) for 72 h. Radiolabeled cDNA was prepared from the mRNA and then hybridized to two identical cytokine gene array membranes. The amount of radioactivity in each spot of the hybridized membranes was determined. Then, the relative changes in mRNA levels in the HG- treated cells were calculated as fold induction over NG-treated cells after normalization to the expression of three internal standards, GAPDH, β-actin, and cyclophilin A.

The results from two independent experiments shown in Fig. 1 and Table 2 indicate that HG treatment upregulated the expression of 41 genes and downregulated 15 genes (Table 2). Interestingly, HG induced the expression of specific groups of genes, including adhesion molecules, chemokines, chemokine receptors, integrins, and TNF superfamily members. We observed marked two- to fourfold increases in mRNA expression of not only cytokines such as TNF-α and IL-1β, but also their receptors: TNF-R and IL-1R, CD27L (related to TNF-R), chemokines MCP-1 and IP-10, chemokine receptors such as CCR-2B, members of TGF-β superfamily, adhesion molecules such as MCAM and PECAM-1, integrins such as β_2-integrin, proteases (MMP-13), protease inhibitors (TIMP-2), and nuclear receptors. IP-10 is a novel IFN-γ–inducible chemokine (19,20) that was recently shown to be increased in diabetic patients (21) and to possess chemotactic effects (22–24). The observed stimulation of these chemokines and integrin expression could be relevant to increased monocyte adhesion, transmigration, and infiltration, as seen in diabetes. Interestingly, many of these genes induced by HG are regulated by the transcription factor NF-κB, suggesting an important regulatory role for NF-κB in hyperglycemia-induced effects. These results show for the first time that multiple inflammatory cytokines, chemokines, integrins, and adhesion molecules are induced under HG conditions in monocytes.

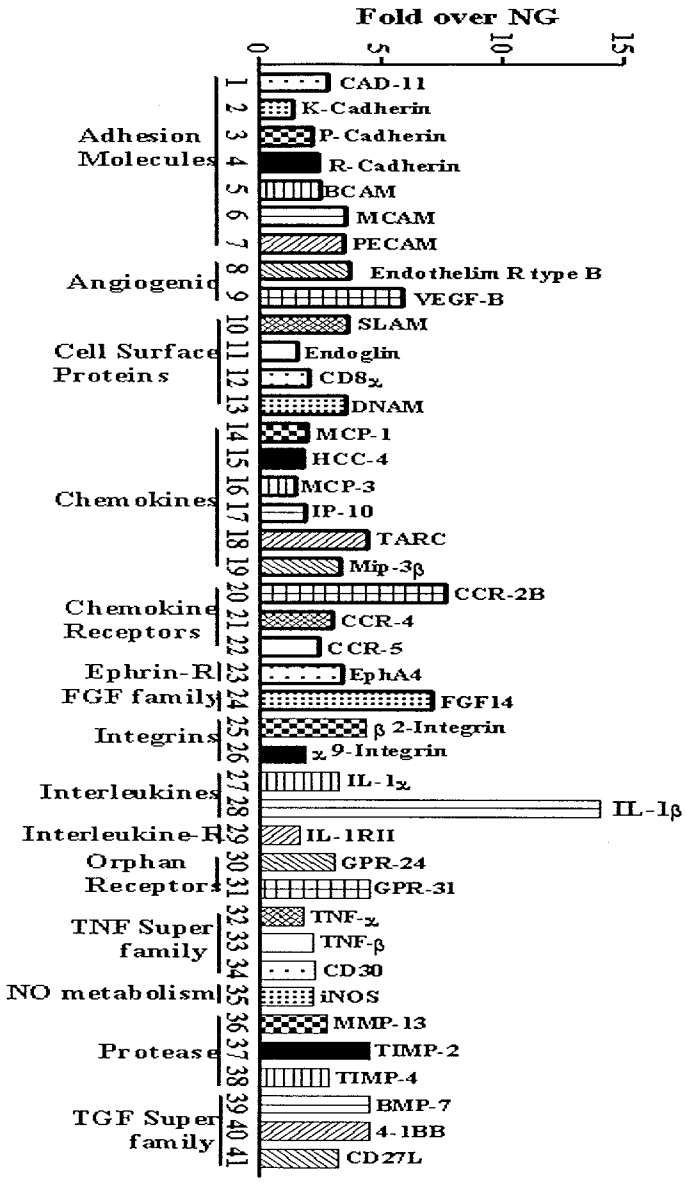

FIG. 1. Gene array analysis of HG-induced expression of cytokine, chemokine, and related genes in THP-1 monocytes. Radiolabeled cDNA from THP-1 monocytes cultured under either NG (5.5 mmol/l) or HG (15 mmol/l) conditions for 72 h were hybridized to human cytokine gene array membranes. Intensity of the spots in the membranes were normalized to internal controls and expressed as fold over NG. All values are average of data from two independent experiments.

HG-induced expression of atherogenic genes. To further confirm the data obtained from the cytokine gene array (Fig. 1), we performed subsequent relative RT-PCR analyses on a subset of genes. Using this method, specific primers for human genes (Table 1) were paired with 18S rRNA primers as internal standards in the multiplex RT-PCRs. RNA extracted from 72-h NG- or HG-treated samples were used in relative RT-PCRs, and HG-induced changes in gene expression were expressed as fold over NG-treated samples after normalizing to 18S rRNA internal control. The results showed that HG increased the expression of MCP-1, IP-10, PECAM-1, β_2-integrin, and IL-1β by two- to fivefold (Fig. 2*A*). In contrast, the expression of β_1-integrin was not significantly affected. These results further confirmed the microarray data. Furthermore, it also demonstrated that HG can induce the expression of key genes relevant to monocyte activation and dysfunction associated with atherosclerosis and other diabetes vascular complications.

We then performed additional RT-PCRs with gene-specific primers (19,25,26) (Table 1) with RNA isolated from several independent experiments to confirm significant regulation of three key genes (MCP-1, IP-10, and β_2 -integrin) that are relevant to monocyte activation. Figure 2*B* shows representative ethidium bromide–stained agarose gels of the RT-PCR products. Figure 2*C* shows fold induction of each gene and demonstrates that HG-induced MCP-1, IP-10, and β_2-integrin mRNAs were statistically significant ($P < 0.01$, $n = 3$–5).

HG regulates the MCP-1 mRNA and protein expression. MCP-1 plays an important role in monocyte chemo-

TABLE 2
HG-induced changes in gene expression in THP-1 cells

Gene name (accession nos.)	(Fold change relative to NG)
Adhesion	
CAD-11 (L34056)	↑ (2.9)
K-cadherin (D31784)	↑ (1.47)
P-cadherin (X63629)	↑ (2.21)
R-cadherin (L34059)	↑ (2.49)
B-CAM (X80026)	↑ (2.52)
PECAM-1 (M28526)	↑ (3.60)
MCAM (M28882)	↑ (3.49)
Angiogenic	
Endothelin R type B (L06623)	↑ (3.8)
VEGF-B (U43368)	↑ (5.9)
VEGF-D (D89630)	↓
Cell surface proteins	
SLAM (U33017)	↑ (3.7)
Endoglin (X72012)	↑ (1.6)
CD8α (M12828)	↑ (2.1)
CD-34 (M81104)	↓
DNAM-1 (U56102)	↑ (3.57)
Chemokines	
MCP-1 (S69738)	↑ (2.0)
HCC-4 (U91746)	↑ (1.8)
MCP-3 (X71087)	↑ (1.5)
IP-10 (X02530)	↑ (1.9)
TARC (D43767)	↑ (4.48)
MIP-3β (AB000887)	↑ (3.39)
Chemokine receptors	
CCR-2B (U03905)	↑ (7.7)
CCR-4 (X85740)	↑ (3.0)
CCR-5 (U57840)	↑ (2.5)
CCR-7 (L31581)	↓
Cytokines	
IGF-1 (X56773)	↓
GM-CSF (M10663)	↓
M-CSF-1 (M64592)	↓
MSP (L11924)	↓
Cytokine receptors	
IFN-α/β Rβ (U29584)	↓
PDGF Rβ (M21616)	↓
Ephrin Receptors	
Eph-A2 (M59371)	↑ (3.4)
Eph-B6 (D83492)	↓
FGF family	
FGF-7 (M60828)	↓
FGF-9 (D14838)	↓
FHF-1 (U66197)	↓
FHF-4 (U66200)	↑ (7.1)
Integrin	
β_2-Integrin (M15395)	↑ (4.4)
α_9-Integrin (L24158)	↑ (1.9)
Interleukin	
IL-1α (M28983)	↑ (3.3)
IL-1β (M15330)	↑ (14)
Int Receptors.	
IL-1RII (U64094)	↑ (1.7)
Orphan Receptors	
GPR24 (U71092)	↑ (3.1)
GPR31 (U65402)	↑ (4.6)
TNF Superfamily	
TNF-α (M10988)	↑ (1.87)
TNF-β (D12614)	↑ (2.2)
CD30 (M83554)	↑ (2.3)
NO metabolism	
iNOS (L09210)	↑ (2.25)
Protease	
MMP-13 (X75308)	↑ (2.8)
TIMP-2 (M32304)	↑ (4.6)
TIMP-4 (U76456)	↑ (2.9)
TGF β Superfamily	
BMP-7 (NM_001719)	↑ (4.6)
4-1BB (U03397)	↑ (4.6)
Apo-2 ligand (U57059)	↓
CD27L (L08096)	↑ (3.3)
Neurotrophic Factor	
GDNF (L19063)	↓

↑, increase in expression; ↓, decrease in expression.

taxis and vascular disease, including atherosclerosis (27–29). However, its role in diabetic vascular disease has not been well explored. Since we observed for the first time that HG could induce MCP-1 mRNA expression in monocytes, we chose to examine it more extensively, including mechanisms of its regulation.

Interestingly, the time course of MCP-1 mRNA induction seen in Fig. 3*A* indicates that it was induced only after 72 h of HG treatment in THP-1 cells. HG-stimulated MCP-1 expression in the presence (FBS) and absence (serum depleted) of serum (Fig. 3*B*). MCP-1 induction in THP-1 cells was specific to glucose since equimolar amounts of mannitol (MN; osmolality control) had no effect (Fig. 3*C*). Furthermore, Fig. 3*D* shows that HG-induced MCP-1 expression was blocked in cells that were pretreated with a TNF-α neutralizing antibody but not a control antibody, suggesting a TNF-α–dependent mechanism for MCP-1 production. The TNF-α antibody had no effect in the NG cells.

We previously showed that HG stimulates expression of TNF-α in THP-1 cells (16). We therefore compared the time course of MCP-1 induction with that of TNF-α by RT-PCR using primers specific for human TNF-α (30). TNF-α mRNA expression was induced at 24 (1.3-fold) and 48 h (2-fold) and remained elevated at 72 h (2.8- to 3-fold) (data not shown). This is in contrast to MCP-1, which was induced only by 72 h (Fig. 3*A*). This further supports the possibility that HG-induced MCP-1 expression is mediated at least in part via initial production of TNF-α.

We then examined the levels of MCP-1 peptide released by THP-1 cells incubated with HG for 72 h. MCP-1 levels in culture supernatants were quantitated by ELISA. Figure 3*E* demonstrates that HG also significantly increased MCP-1 secretion (more than sevenfold, $P < 0.001$). Thus, HG treatment significantly increased MCP-1 mRNA as well as protein expression.

NF-κB–dependent activation of MCP-1 promoter by HG. We next determined whether HG-induced MCP-1 expression was transcriptionally regulated with a reporter plasmid, p420-MCP-1-Luc, in which luciferase gene is under the control of a minimal human MCP-1 promoter (−420 to 37). This promoter fragment contains key NF-κB

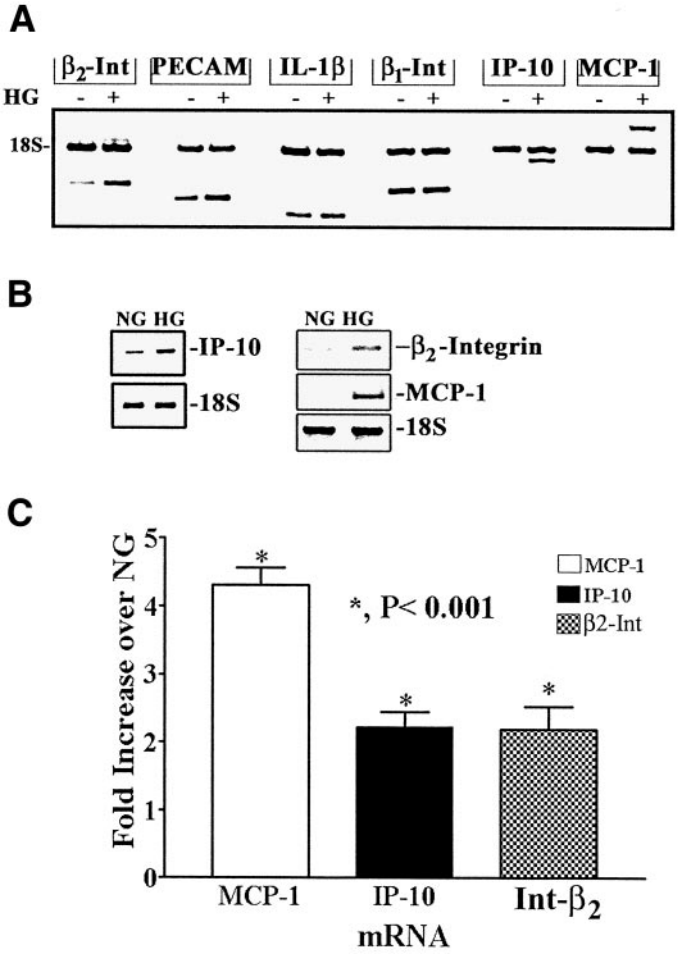

FIG. 2. ***A*****: Analyses of HG-induced gene expression by relative multiplex RT-PCR. Relative multiplex RT-PCRs were performed with total RNA isolated from THP-1 cells treated under NG and HG conditions for 72 h using gene-specific primers (listed in Table 1). 18S RNA primers were included in the same reaction as internal control. PCR products were analyzed on 2.5% agarose gels. Figure shows agarose gel of RT-PCR products. Signs "−" and "+" above the lanes indicate NG- and HG-treated cells, respectively.** ***B*** **and** ***C*****: HG-treated THP-1 cells show significant increases in the mRNA levels of MCP-1, IP-10, and β_2-integrin. Total RNA from multiple sets of NG- and HG-treated THP-1 cells were used to perform relative multiplex RT-PCRs using gene-specific primers and 18S RNA primers (Fig.** ***B*****). The intensity of each gene-specific band was normalized to internal control (18S RNA) and results expressed as fold stimulation over NG (Fig.** ***C*****). Values shown are mean ± SE of three to five independent experiments. *$P < 0.001$.**

binding regions that are known to play a role in MCP-1 transcription (31,32). THP-1 cells were transiently transfected with p420-MCP-1-Luc or the pCR-Luc (control vector lacking MCP-1 promoter), the transfected cells grown in NG or HG conditions for 72 h, and luciferase activity determined. The results (Fig. 4*A*) showed that in THP-1 cells transfected with p420-MCP1-Luc, HG treatment led to a fivefold increase in luciferase activity compared with NG. In contrast, there was no change in cells transfected with control vector. These results indicate that HG can lead to transcriptional regulation of MCP-1 and that some of the key *cis* elements involved are in the −420 region of the promoter.

Since this promoter fragment also contains one NF-κB binding site (−90 to −81), we evaluated the role of NF-κB in HG-induced transcriptional response. THP-1 cells were cotransfected with p420-MCP-1-Luc along with pCMV-mIκB plasmid, which expresses an IκBα "super-repressor" mutant (serine 32 and 36 substituted by alanine residues) that blocks NF-κB activation by inhibiting the phosphorylation and degradation of IκBα (18). Transfected cells were treated with HG for 72 h and luciferase activity determined. As shown in Fig. 4*B*, cells transfected with p420-MCP-1-Luc alone showed a significant increase in luciferase activity under HG conditions relative to NG as in Fig. 4*A*. In contrast, cotransfection with the mIκB plasmid significantly attenuated HG stimulation of MCP-1 promoter. The mIκB vector had no significant effect on control pCR-Luc plasmid under basal or HG-treated conditions. These results indicate that NF-κB activation plays an important role in HG-induced MCP-1 transcription in these cells.

Signal transduction mechanisms involved in HG-induced MCP-1 mRNA expression. Several lines of evidence suggest that the pathological effects of HG can be mediated by the activation of superoxide production (from NADPH oxidase and mitochondria), PKC, and MAPKs such as p38MAPK and ERK1/2 (10,16,17,33–35). To determine the role of these key pathways in HG-induced MCP-1 mRNA expression, we evaluated the effects of specific inhibitors of these pathways. The results in Fig. 5*A* indicate that HG effects on MCP-1 mRNA expression were blocked by inhibitors of p38MAPK (SB202190, SB), PKC (GF), ERK1/2 MAPK (PD98059, PD), a mitochondrial electron transport complex II inhibitor (TTFA), and an antioxidant NAC. In addition, Fig. 5*B* indicates that HG-induced MCP-1 was also blocked by the NADPH oxidase inhibitor, apocynin, suggesting that superoxide arising from NADPH oxidase as well as mitochondria may be involved under these conditions. A recent study demonstrated the key role of PKC-α and NAPDH oxidase in HG-induced superoxide production in THP-1 cells (35). Our results indicate that multiple pathways that may act in unison are involved in HG-induced MCP-1 expression.

HG-induced adhesion of monocytes to HAECs. To evaluate the functional significance of the induction of

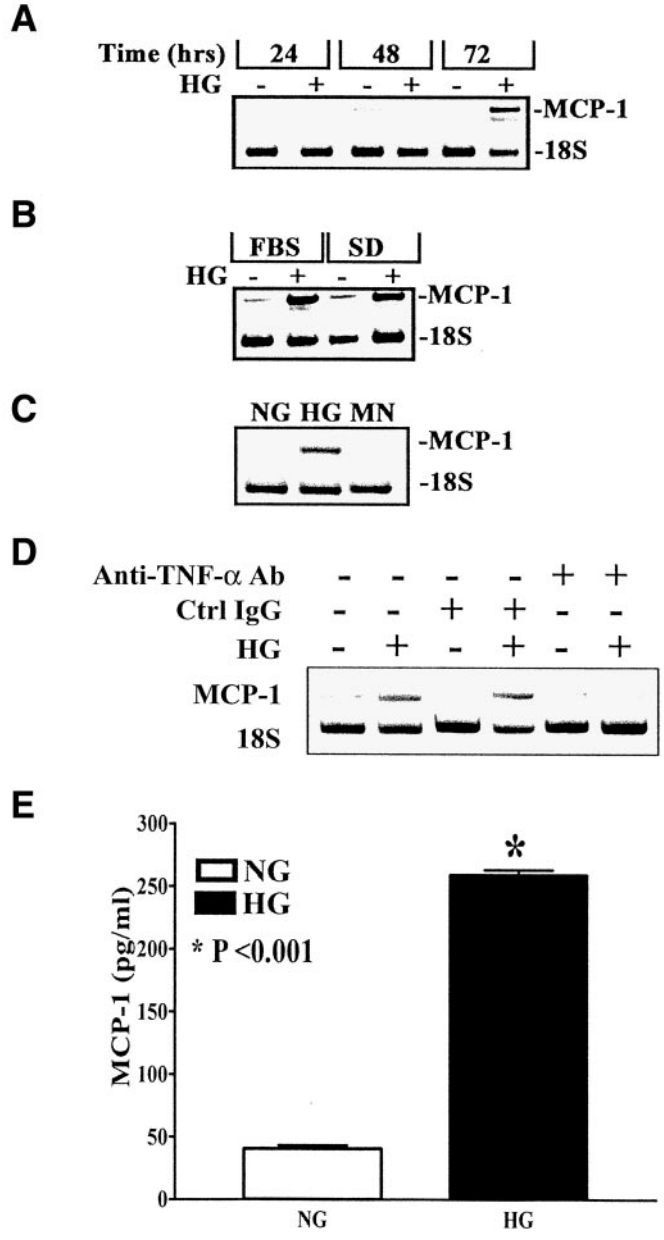

FIG. 3. Characterization of HG-induced MCP-1 mRNA and protein expression. *A*: THP-1 cells were cultured under NG or HG conditions in serum-containing medium for 24, 48, and 72 h and RT-PCR performed as described in Fig. 2. *B*: Cells were cultured for 72 h in serum-containing medium (FBS) or in serum-depleted (SD) medium containing 0.2% BSA (SD). Signs "−" and "+" above the lanes indicate without or with HG, respectively. *C*: THP-1 cells were treated with either MN (9.5 mmol/l) or HG (15 mmol/l) for 72 h as in *A*. Total RNA was isolated and RT-PCR performed with MCP-1 specific primers and 18S primers. *D*: THP-1 cells were pretreated with TNF-α antibody (50 ng/ml) or control IgG (50 ng/ml) for 1 h and then treated with HG (15mmol/l) for 72 h. Total RNA isolation and RT-PCR were performed as described above. *E*: Conditioned medium supernatants of THP-1 cells treated with NG or HG were assayed for secreted MCP-1 levels by ELISA using a specific MCP-1 antibody, as described in RESEARCH DESIGN AND METHODS. Results shown are mean ± SE from three experiments run in triplicate.

multiple monocyte activating genes, we examined whether HG treatment of monocytes can increase their adhesion to HAECs. THP-1 cells were cultured in NG or HG medium with serum for 48 h, followed by incubation in serum-depleted medium for 24 h, and then allowed to adhere to normal HAECs. As seen in Fig. 6*A*, THP-1 cells cultured in HG could adhere to a fourfold greater extent to HAECs ($P < 0.005$), as compared with cells in NG. Interestingly, we also observed that treatment of the THP-1 cells, even for 24 h with HG, could also increase their adherence to HAECs (2.5 ± 0.03-fold over NG, $P < 0.01$), although this effect was lesser than the 3-day HG treatment.

Identification of signaling pathways involved in HG-induced monocyte adhesion. Our recent studies have demonstrated that hyperglycemia-induced monocyte activation involves the activation of oxidant stress–dependent as well as –independent pathways (16). HG could increase the production of superoxide and activate the oxidant stress-responsive transcription factors NF-κB and AP-1, as well as the MAPKs p38 and ERK1/2 (16). Earlier evidence also indicates that several pathological effects of HG are associated with PKC activation and mitochondrial superoxide production (10,17). We therefore examined the consequences of blocking some of these signaling pathways on HG-induced monocyte adhesion to determine their functional role. In these studies, adhesion to HAEC was determined with THP-1 cells cultured for 24 h in HG with or without specific inhibitors. Figure 6*B* shows that HG-induced monocyte binding to HAEC was significantly blocked by the antioxidant PDTC (100 μmol/l), as well as by the PKC (GF, 100 nmol/l) and p38MAPK (SB, 1 μmol/l) inhibitors. These results suggest that HG-induced monocyte activation and adhesion involve the coordinated activation of multiple pathways, including oxidant stress, PKC, and p38MAPK. These pathways may operate via induction of key cytokine and chemokine genes, many of which are NF-κB regulated.

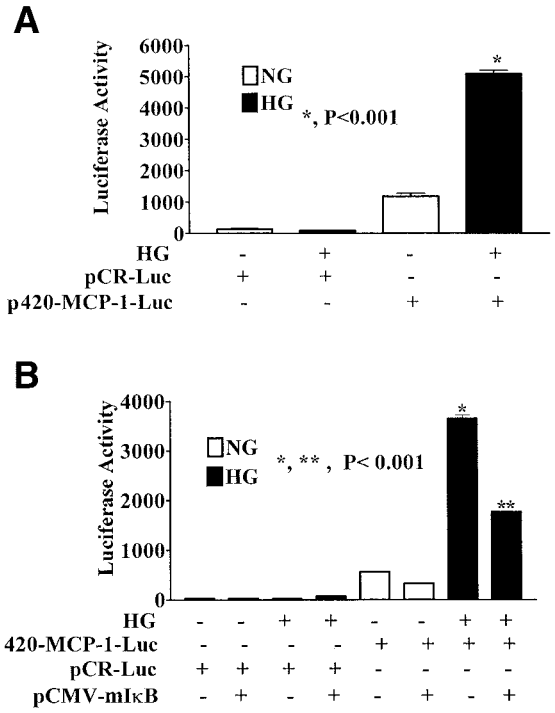

FIG. 4. ***A*****: Stimulation of MCP-1 promoter by HG. THP-1 cells were transfected with either a control plasmid containing the promoter-less luciferase gene pCR-Luc or a plasmid p420-MCP1-Luc that contains luciferase gene under the control of a minimal human MCP-1 promoter (−427 to 37). After a 24-h recovery period, cells were treated with HG for 72 h, lysed, and luciferase activity determined using a luminometer. Values shown are Luciferase activity normalized to 100 μg protein (mean ± SE of three independent experiments) ****P*** **< 001 vs. NG.** ***B*****: Involvement of NF-κB in HG-stimulated MCP-1 promoter activation in THP-1 cells. THP-1 cells were transfected with either a control plasmid (pCR-Luc) or a plasmid with MCP-1 promoter (p420-MCP1-Luc), as described in Fig. 5*A*. In addition, some cells were cotransfected with a plasmid containing a mutant IκB expressed from the CMV promoter (pCMV-mIκB) that can repress NF-κB activation. After 24 h recovery, cells were treated with HG for 72 h, lysed, and luciferase activities determined. Values shown are luciferase activity normalized to 100 μg protein (mean ± SE of three independent experiments). ****P*** **< 0.001 vs. NG; *****P*** **< 0.001 vs. HG.**

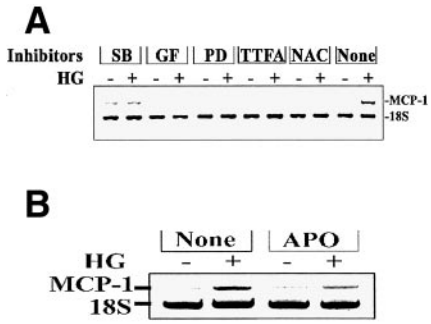

FIG. 5. ***A*** **and** ***B*****: Effect of kinase inhibitors and antioxidants on HG-induced MCP-1 mRNA. THP-1 cells were treated with or without the indicated inhibitors in NG or HG medium for 72 h. Total RNA was isolated and RT-PCR performed using MCP-1 specific primers and 18S primers. Signs "−" and "+" above the lanes indicate without or with HG, respectively. APO, apocynin (NADPH oxidase inhibitor); GF, bis-indolylmaleimide (PKC inhibitor); None, MCP-1 expression without any inhibitor; SB, SB202190 (p38MAPK inhibitor). TTFA is a mitochondrial complex II inhibitor, and NAC is an antioxidant.**

DISCUSSION

It is well known that inflammatory cytokines and chemokines such as TNF-α, IL-1β, and MCP-1 play important roles in monocyte activation and the pathogenesis of atherosclerosis (27–29,36). In the present studies, we performed DNA array analyses to demonstrate for the first time that HG treatment of THP-1 monocytes induced changes in the expression levels of multiple cytokines, chemokines, and related molecules. Subsequently, we confirmed the expression of key genes by RT-PCR and ELISA analyses. We noted marked increases (two- to fivefold) in mRNA expression of cytokines such as TNF-α and IL-1β, their receptors (TNFR and IL-1R), CD27L, and chemokines such as MCP-1 and the potent inflammatory and chemotactic gene IP-10. There were also clear increases in the expression of the angiogenic and vascular permeability factors (vascular endothelial growth factors), inhibitors of metalloproteinases (TIMP-2 and -4), and the metalloproteinase MMP-13. The expression of β_2-integrin was also upregulated. Many of these genes induced by HG are known to be NF-κB regulated. It therefore appears that HG-induced activation of NF-κB has far-reaching consequences in leading to the induction of multiple genes known to mediate monocyte activation and the pathogenesis of atherosclerosis.

We observed for the first time in monocytes that HG significantly increased the expression of MCP-1, a chemoattractant member of the chemokine superfamily. The effects were specific to HG since MN had no effect. MCP-1 is implicated in inflammatory responses, the pathogenesis of atherosclerosis, immune regulation, wound healing, tissue remodeling, and modulation of tumor behavior (27,37). In human monocytes, MCP-1 induces chemotaxis, calcium flux, and respiratory burst, as well as upregulates adhesion molecule expression and cytokine production (27). A wide variety of activated cells, including monocytes, fibroblasts, vascular endothelial cells, and smooth muscle cells produce MCP-1 in vitro in response to various stimuli such as lipopolysaccharide (LPS), IL-1β, and TNF-α. (27–29,37). Studies in vitro and in vivo with MCP-1 knockout mice have shown its importance in the development of atherosclerosis (28,29). The actions of MCP-1 are thought to be related to its chemotactic effects responsible for monocyte recruitment in acute and chronic inflammatory states (38). MCP-1 itself is capable of inducing IL-1β, TNF-α, and IP-10 in macrophages (39). These results indicate that MCP-1 is not only a chemoattractant but also a novel cytokine with the capacity to regulate several parameters of monocyte function. Our data showing that HG induces NF-κB–dependent transcriptional regulation of MCP-1 adds to the growing list of HG-induced NF-κB–regulated inflammatory genes. We also noted for the first time that HG increased the expression of TNF-α temporally before MCP-1 and that a TNF-α neutralizing antibody blocked HG-induced MCP-1 mRNA expression. This raises the interesting possibility that the induction of inflammatory genes by HG may be both direct, as well as indirect, via vicious feedback and feed-forward loops where the initially formed genes can autoregulate themselves and each other.

Our gene array analyses also came up with another interesting hit, namely IP-10, a CXC chemokine that is induced in response to interferon stimulation (19) and regulated, at least in part, by NF-kB (40,41). Human IP-10 mRNA is highly induced in differentiated human monocytic U937 cells by IFN-γ (42). It is also induced by TNF-α

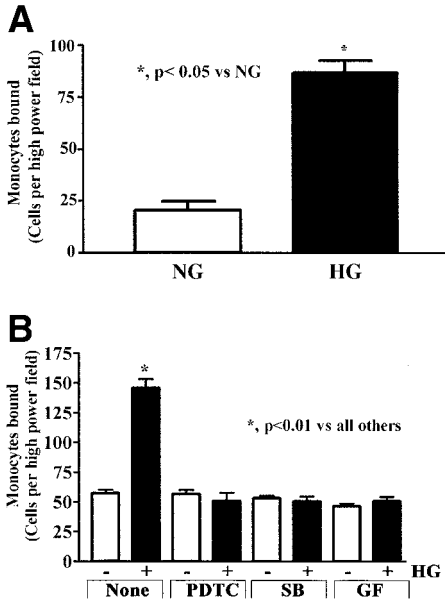

FIG. 6. *A*: HG culture of monocytes increases their adhesion to HAECs. THP-1 cells were cultured in HG for 72 h and allowed to adhere to HAECs plated in 48-well culture dishes. After careful washing, bound monocytes were fixed and counted. Results are expressed as number of monocytes bound per high-power field. Results represent mean ± SE from 10 experiments. *P < 0.005 vs. control. *B*: Effect of inhibitors on HG-induced monocyte adhesion. THP-1 cells were treated for 24 h with the indicated inhibitors in NG or HG medium, followed by binding to HAECs (examined as in Fig. 6*A*). Results shown are mean ± SE (n = 5). *P < 0.01 vs. control and all the three inhibitors shown. GF, bisindolylmaleimide; SB, SB202190.

(39,43,44), again suggesting a potential intercytokine regulatory mechanism. IP-10 regulation by HG is also relevant since it is a potent inflammatory chemokine for monocytes and vascular smooth muscle cells (22,23). IP-10 has also been implicated in islet cell destruction (45) and was recently shown to be increased in diabetic patients (21), thus raising the intriguing possibility that it could serve as a novel inflammatory marker for diabetes complications.

Earlier studies have demonstrated that HG culture of endothelial cells could increase their binding to monocytes (14). Our present data demonstrate for the first time that HG culture of monocytes can also increase their adhesion to HAEC. Furthermore, our mechanistic studies suggest the involvement of PKC, oxidant stress, and p38MAPK, pathways that are also activated by HG (10,16,17,33–35). Thus, HG-induced monocyte activation involves the coordinated activation of multiple pathways that can lead to the induction of key inflammatory genes, many of which are NF-κB regulated and could also regulate each other in novel autocrine/paracrine loops. Thus, one of the key functional consequences of HG-induced expression of inflammatory genes and adhesive integrin receptors is increased monocyte adhesion and activation. These mechanisms could be responsible for the accelerated inflammation and atherosclerosis observed under diabetic conditions.

In the present study, we treated monocytes with HG for 3 days since this is the normal in vivo turnover time for monocytes. In the diabetic state in vivo, it is clear that advanced glycation end products can contribute to and augment the inflammatory gene expression induced by HG. Furthermore, in type 2 diabetes, there can be an added effect of hyperinsulinemia and hyperlipidemia. Studies with monocytes from diabetic patients have indicated increased adhesion to endothelial cells relative to those from normal individuals (13). Human diabetic monocytes have increased NF-κB activity (46,47). Our in vitro studies are in agreement with these in vivo studies and, in addition, provide mechanistic data for these clinical observations. Furthermore, the data and hits obtained from our gene array analyses demonstrate the relevance to not only cardiovascular disorders, but also to other complications and metabolic defects associated with diabetes. For example, increases in monocyte MMP-13 could be associated with plaque instability, vascular endothelial growth factor increase could be related to diabetic retinopathy, cytokine and chemokine increase could be associated with macrophage infiltration and islet destruction, and TIMP increase may be related to the increased extracellular deposition noted in diabetic nephropathy. Overall, our studies indicate that HG can trigger a cascade of genes involved in the pathogenesis of cardiovascular as well as other complications of diabetes.

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