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, chemokine 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, β₂-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 and certain key pathways, factors, and mechanisms have been implicated, including oxidant stress, advanced glycation end products, aldose reductase, and protein kinase C (PKC) activity. 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. 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. 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 β₂-integrins. 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. HG culture also increased basal and TNF-α–induced p38 mitogen-activated protein kinase (MAPK) activity in these monocytes. 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 β₂-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-indolylmethylamine, and N-acetylcysteine (NAC) were purchased from Calbiochem (San Diego, CA); PD98059 from Cell Signaling (Beverly, MA); and pyrrolidine-2-thione dihydrogen succinate (TTFA).
dithiocarbamate (PDTC), thieno[3,2-d]thiazole-4-carboxylate (TTFA), and apocynin from Sigma-Aldrich Chemicals (St. Louis, MO). Human panorama cytokine gene arrays and corresponding reagents were from Sigma-Aldrich Chemicals.

\[^{32}\text{P}]\text{dCTP} (3,000 \text{ 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 Oligotex kits were from Qiagen (Valencia, CA). Luciferase assay system was from Promega (Madison, WI).

**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 \(\mu\text{g} / \text{ml}\)), apocynin (30 \(\mu\text{mol/l}\)), NAC (thiol antioxidant that also serves as a glutathione precursor, 100 \(\mu\text{mol/l}\)), PDTC (antioxidant, 100 \(\mu\text{mol/l}\)), PD98059 (ERK MAPK pathway inhibitor, 0.5 \(\mu\text{mol/l}\)), and normal rabbit IgG were from R&D Systems (Minneapolis, MN).

**DNA transfection and luciferase assays.** THP-1 cells plated in a six-well plate (1.2 \(\times\ 10^5\) per well) were transfected with 1 \(\mu\text{g}\) 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 β-gal 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 24 h when NG or HG medium, washed with PBS, lysed with 100 \(\mu\text{l}\) of lysis buffer, and stored overnight at −70°C. Samples were thawed, brought to room temperature, and 20 \(\mu\text{l}\) of each lystate used to analyze luciferase activity.

**Measurement of MCP-1 levels by ELISA.** Supernatants of THP-1 cells (5 \(\times\ 10^5\) 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.

**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 β2-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.

**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. All values are average of data from two independent experiments.

The observed stimulation of MCP-1, IP-10, PECAM-1, and PECAM-2 integrin 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.](image)

**HG regulates the MCP-1 mRNA and protein expression.** MCP-1 plays an important role in monocyte chemo-
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. 3A 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. 3B). MCP-1 induction in THP-1 cells was specific to glucose since equimolar amounts of mannitol (MN; osmolality control) had no effect (Fig. 3C). Furthermore, Fig. 3D 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. 3A). 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 3E 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

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**TABLE 2**

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

<table>
<thead>
<tr>
<th>Gene name (accession nos.)</th>
<th>(Fold change relative to NG)</th>
<th>Gene name (accession nos.)</th>
<th>(Fold change relative to NG)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adhesion</strong></td>
<td></td>
<td><strong>Ephr Receptors</strong></td>
<td></td>
</tr>
<tr>
<td>CAD-11 (L34056)</td>
<td>↑ (2.9)</td>
<td>Eph-A2 (M59371)</td>
<td>↑ (3.4)</td>
</tr>
<tr>
<td>K-cadherin (D31784)</td>
<td>↑ (1.47)</td>
<td>Eph-B6 (D83492)</td>
<td>↓</td>
</tr>
<tr>
<td>P-cadherin (X65628)</td>
<td>↑ (2.21)</td>
<td>FGF family</td>
<td></td>
</tr>
<tr>
<td>R-cadherin (L34059)</td>
<td>↑ (2.49)</td>
<td>FGF-7 (M60628)</td>
<td>↓</td>
</tr>
<tr>
<td>B-CAM (X80026)</td>
<td>↑ (2.52)</td>
<td>FGF-9 (D14838)</td>
<td>↓</td>
</tr>
<tr>
<td>PECAM-1 (M28526)</td>
<td>↑ (3.60)</td>
<td>FHF-1 (U66197)</td>
<td>↓</td>
</tr>
<tr>
<td>MCAM (M28882)</td>
<td>↑ (3.49)</td>
<td>FHF-4 (U66209)</td>
<td>↑ (7.1)</td>
</tr>
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<td><strong>Angiogenic</strong></td>
<td></td>
<td><strong>Integrin</strong></td>
<td></td>
</tr>
<tr>
<td>Endothelin R type B (L06623)</td>
<td>↑ (3.8)</td>
<td>β2-Integrin (M15395)</td>
<td>↑ (4.4)</td>
</tr>
<tr>
<td>VEGF-B (U43368)</td>
<td>↑ (5.9)</td>
<td>α5-Integrin (L24158)</td>
<td>↑ (1.9)</td>
</tr>
<tr>
<td>VEGF-D (D89630)</td>
<td>↓</td>
<td><strong>Interleukin</strong></td>
<td></td>
</tr>
<tr>
<td>Cell surface proteins</td>
<td></td>
<td>IL-1α (M28983)</td>
<td>↑ (3.3)</td>
</tr>
<tr>
<td>SLAM (U33017)</td>
<td>↑ (3.7)</td>
<td>IL-1β (M15530)</td>
<td>↑ (14)</td>
</tr>
<tr>
<td>Endoglin (X72012)</td>
<td>↑ (1.6)</td>
<td>Int Receptors.</td>
<td></td>
</tr>
<tr>
<td>CD8α (M12828)</td>
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<td>IL-1RII (U64094)</td>
<td>↑ (1.7)</td>
</tr>
<tr>
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<td>↓</td>
<td>Orphan Receptors.</td>
<td></td>
</tr>
<tr>
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<td>GPR24 (U17092)</td>
<td>↑ (3.1)</td>
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<td><strong>Chemokines</strong></td>
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<td>GPR31 (U65402)</td>
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<tr>
<td>MCP-1 (S69738)</td>
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<td>TNF Superfamily</td>
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<td>TNF-α (M10988)</td>
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</tr>
<tr>
<td>MCP-3 (X71087)</td>
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<td>TNF-β (D12614)</td>
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</tr>
<tr>
<td>IP-10 (X02530)</td>
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<td>CD30 (M83554)</td>
<td>↑ (2.3)</td>
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<tr>
<td>TARC (D43767)</td>
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<td>NO metabolism</td>
<td></td>
</tr>
<tr>
<td>MIP-3β (AB000887)</td>
<td>↑ (3.39)</td>
<td>iNOS (L09210)</td>
<td>↑ (2.25)</td>
</tr>
<tr>
<td><strong>Chemokine receptors</strong></td>
<td></td>
<td><strong>Protease</strong></td>
<td></td>
</tr>
<tr>
<td>CCR-2B (U03905)</td>
<td>↑ (7.7)</td>
<td>MMP-13 (X75308)</td>
<td>↑ (2.8)</td>
</tr>
<tr>
<td>CCR-4 (X85740)</td>
<td>↑ (3.0)</td>
<td>TIMP-2 (M23204)</td>
<td>↑ (4.6)</td>
</tr>
<tr>
<td>CCR-5 (U57840)</td>
<td>↑ (2.5)</td>
<td>TIMP-4 (U76456)</td>
<td>↑ (2.9)</td>
</tr>
<tr>
<td>CCR-7 (L31581)</td>
<td>↓</td>
<td>TGF β Superfamily</td>
<td></td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td>BMP-7 (NM_001719)</td>
<td>↑ (4.6)</td>
</tr>
<tr>
<td>IGF-1 (X56773)</td>
<td>↓</td>
<td>4-1BB (U03937)</td>
<td>↑ (4.6)</td>
</tr>
<tr>
<td>GM-CSF (M10663)</td>
<td>↓</td>
<td>Apo-2 ligand (U57059)</td>
<td>↓</td>
</tr>
<tr>
<td>M-CSF-F1 (M64592)</td>
<td>↓</td>
<td>CD27L (L08096)</td>
<td>(3.3)</td>
</tr>
<tr>
<td>MSP (L1992)</td>
<td>↓</td>
<td><strong>Neurotrophic Factor</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Cytoine receptors</strong></td>
<td></td>
<td>GDNF (L19063)</td>
<td>↓</td>
</tr>
</tbody>
</table>

↑, increase in expression; ↓, decrease in expression.
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. 4A) 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 IκB "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. 4B, cells transfected with p420-MCP-1-Luc alone showed a significant increase in luciferase activity under HG conditions relative to NG as in Fig. 4A. 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. 5A 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. 5B 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 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. 4A) 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.

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HG-induced adhesion of monocytes to HAECs. To evaluate the functional significance of the induction of
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. 6A, 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 6B 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. 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 (15 mmol/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.
HYPERGLYCEMIA INDUCED GENE EXPRESSION

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 β2-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 chemotactic 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 chemotaxant 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-κB (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.

DISCUSSION

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 pg protein (mean ± SE of three independent experiments) *P < 0.001 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. 5A. 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 pg 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). TFFA is a mitochondrial complex II inhibitor, and NAC is an antioxidant.
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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