

Hyperglycemia Induces Monocytic Release of Interleukin-6 via Induction of Protein Kinase C- α and - β

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Diabetes confers an increased propensity to atherosclerosis. Inflammation is pivotal in atherogenesis, and diabetes is a proinflammatory state. Interleukin (IL)-6, in addition to inducing the acute-phase response, contributes to insulin resistance. Monocytes from type 2 diabetic patients secrete increased IL-6. The aim of this study was to examine molecular mechanisms for increased IL-6 release from monocytes under hyperglycemia. Monocytic cells (THP-1) were cultured in the presence of 5.5 mmol/l (normal) or 15 mmol/l (high) glucose and mannitol. Secreted IL-6, intracellular IL-6, and IL-6 mRNA were significantly increased with hyperglycemia ($P < 0.001$). Incubation of cells with inhibitors of reactive oxygen species failed to affect high-glucose-induced IL-6 release. Pan-protein kinase C (PKC) inhibitors significantly decreased high-glucose-induced IL-6 release. A specific inhibitor of p38 mitogen-activated protein kinase (MAPK; SB 202190), but not the extracellular signal-regulated kinase inhibitor PD98059, significantly decreased high-glucose-induced IL-6 release. Furthermore, the PKC- α/β 2 inhibitor decreased p38MAPK and the resulting high-glucose-induced IL-6 release. Both antisense oligos to PKC- β and - α as well as small interfering RNA (siRNA) to PKC- α and - β resulted in significantly decreased high-glucose-induced IL-6 release. Nuclear factor- κ B (NF- κ B) inhibitors significantly decreased IL-6 mRNA and protein. siRNA to PKC- β and - α also significantly decreased NF- κ B activity and IL-6 release. The combination was not additive to either siRNA alone, suggesting that they work through a common pathway. Thus, IL-6 release from monocytes under hyperglycemia appears to be mediated via upregulation of PKC, through p38MAPK and NF- κ B, resulting in increased mRNA and protein for IL-6. Thus, inhibition of PKC- α and - β can ameliorate the proinflammatory state of diabetes. *Diabetes* 54:85–91, 2005

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Received for publication 30 July 2004 and accepted in revised form 21 September 2004.

DPI, diphenylene iodonium chloride; ERK, extracellular signal-regulated kinase; EMSA, electrophoretic mobility shift assay; HBDDE, 2,2',3,3',4,4'-hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether; IL, interleukin; MAPK, mitogen-activated protein kinase; MCP-1, macrophage chemoattractant protein 1; NF- κ B, nuclear factor- κ B; PEG-SOD, polyethylene glycol-superoxide dismutase; PKC, protein kinase C; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin; siRNA, small interfering RNA; TNF- α , tumor necrosis factor- α .

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Cardiovascular disease is the leading cause of morbidity and mortality in the U.S. The diabetic state confers an increased propensity to accelerated atherosclerosis. Inflammation plays a pivotal role in atherogenesis (1–4). Type 2 diabetes has been postulated as a disease of the innate immune system (5,6). Evidence for increased inflammation in diabetes include increased levels of the prototypic inflammatory marker C-reactive protein as well as the proinflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin (IL)-6 (6–8). Among the proinflammatory cytokines, IL-6 plays a major role in diabetes and cardiovascular disease (9–17). Many studies have shown increased plasma levels of IL-6 in subjects with unstable angina compared with those with stable angina or healthy subjects (10). In several prospective studies, subjects with IL-6 levels in the top quantile had a twofold increased risk of death and cardiovascular events (11–14). Furthermore, IL-6 mRNA and protein have been found to be increased in atherosclerotic lesions (15). Also, Huber et al. (16) have shown that injection of recombinant IL-6 exacerbates atherosclerosis in mice. IL-6 is the major inducer of the acute-phase response, e.g., C-reactive protein and serum amyloid A release (5,6,9), and promotes insulin resistance (5,6,9). Diabetic subjects have increased levels of plasma IL-6 (7). We have shown that monocytes from patients with type 2 diabetes secrete increased amounts of IL-6 (7), and Jain et al. (17) have reported elevated levels of IL-6 in type 1 diabetic subjects. Thus, the aim of this study was to examine potential molecular mechanisms for increased IL-6 release from monocytes under hyperglycemia.

RESEARCH DESIGN AND METHODS

Cells from the human monocytic cell line THP-1 were obtained from the American *Type Culture* Collection. Endotoxin- and glucose-free RPMI-1640 media and fetal bovine serum were purchased from Gibco (Carlsbad, CA). Antibiotics, glutamine, phenylmethylsulfonyl fluoride, glucose, HEPES, protease inhibitor cocktail, Triton X-100, dithiothreitol, polyethylene glycol-superoxide dismutase (PEG-SOD), and Mn(III)tetrakis(4-benzoic acid) porphyrin (MnTBAP), were from Sigma. Antibodies to PKC, PKC- α , and PKC- β II, were obtained from Santa Cruz Biotechnology. 2,2',3,3',4,4'-hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether (HBDDE), apocynin, and diphenylene iodonium chloride (DPI) were obtained from Calbiochem, and Eli Lilly kindly provided LY379196. Polyvinylidene difluoride membranes and Tris-glycine gels were from Invitrogen. A bicinchoninic acid kit was obtained from Pierce. Enhanced chemiluminescence and PKC activity kits were purchased from Amersham Pharmacia. Oligonucleotides were purchased from Integrated DNA Technologies.

Cell culture. THP-1 cells were maintained in endotoxin-free RPMI-1640 (containing 5.5 mmol/l glucose, 50 μ mol/l mercaptoethanol, 10% fetal bovine serum, 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, and 10 mmol/l HEPES) and used for experiments between the third and fifth passages, as described

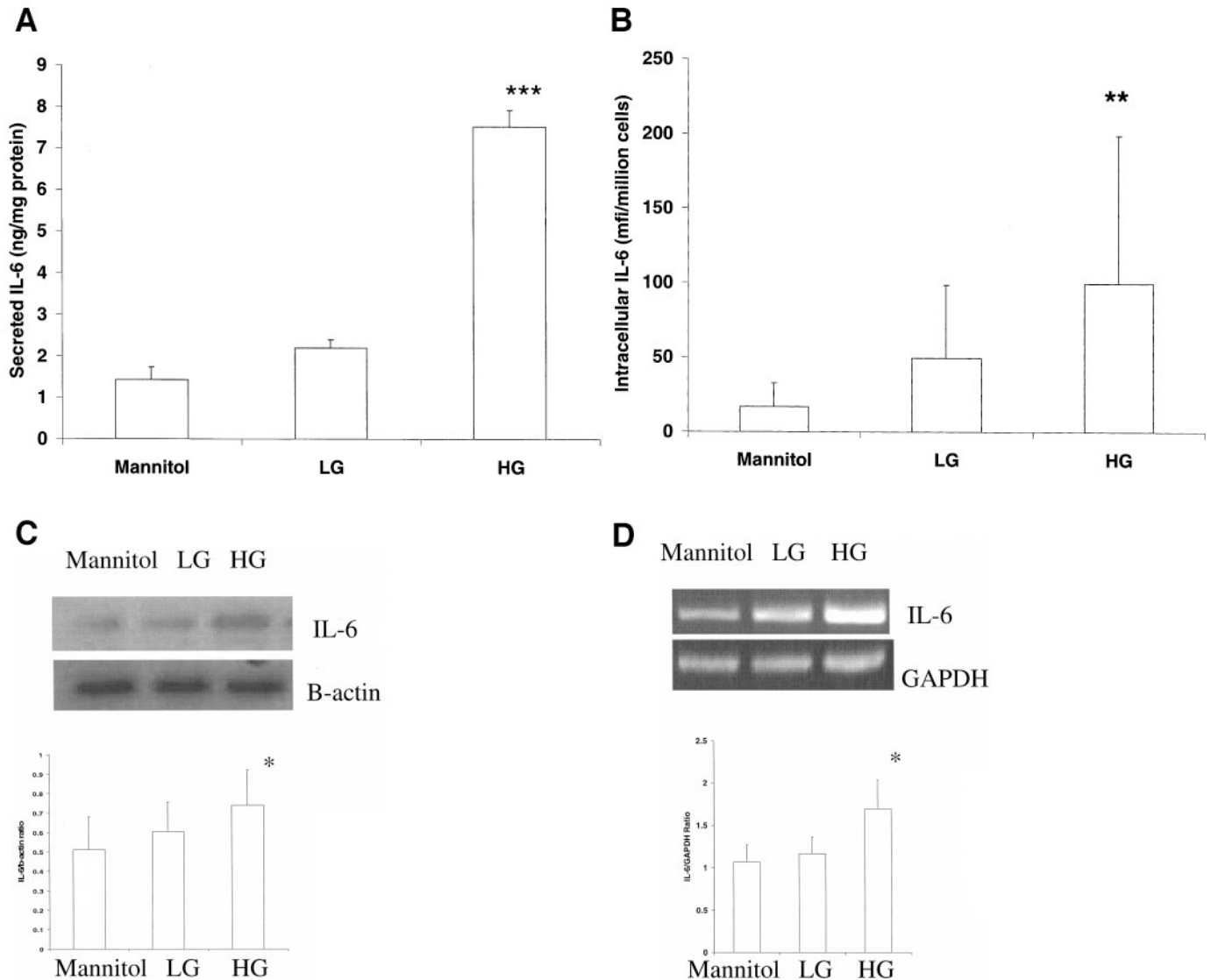


FIG. 1. Effect of hyperglycemia on IL-6 release from THP-1 cells. Cells were cultured in 5.5 mmol/l (LG) and 15 mmol/l (HG) glucose, and IL-6 release was measured as described in RESEARCH DESIGN AND METHODS. As a control, 9.5 mmol/l mannitol was added with normal glucose in simultaneous wells ($n = 5$). **A:** Secreted IL-6 by enzyme-linked immunosorbent assay. **B:** Intracellular IL-6 by flow cytometry. **C:** Western blot for IL-6 using β -actin as control. **D:** RT-PCR for IL-6 using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control. Data are means \pm SD, and asterisks denote significant differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with normal glucose.

previously (15). Cells were cultured (1×10^6 cells/ml) for 2 days in either 5.5 mmol/l (normal glucose) or 15 mmol/l (high glucose) glucose, and as an osmotic control, 9.5 mmol/l mannitol was added along with normal glucose. Although IL-6 release was significantly increased at 24 h after high glucose, we chose a 48-h incubation with glucose for subsequent experiments because we wanted to better mimic the diabetic milieu, where there is not an acute but a chronic elevation of glucose. Cell viability, as determined by trypan blue exclusion, was $>92\%$. Inhibitors were added to cells with normal glucose/high glucose, with daily changes in media.

Measurement of IL-6. Secreted IL-6 release was measured by enzyme-linked immunosorbent assay, using the human immunoassay kit (High Sensitivity Kit; R&D) (5). Also, intracellular IL-6 was measured by flow cytometry after incubation of treated cells with fluorescein isothiocyanate-labeled anti-human IL-6, as described previously (16). Results were expressed as nanomoles per milligram cell protein.

Determination of PKC activity. PKC activity in THP-1 cells was determined by radioimmunoassay (18). It was based on the PKC-catalyzed transfer of the γ -phosphate group of ATP to a PKC-specific peptide. PKC activity was expressed as nanomoles of phosphate transferred per million cells.

Western blotting. At the end of culture, cells were lysed, and membrane fractions were isolated by the method of Venugopal et al. (18), as described previously. Membrane proteins (10–30 μ g) were resolved in 10% Tris-glycine

gel, and blotting was performed with specific primary and secondary antibodies. Blots were visualized by enhanced chemiluminescence detection system.

Electrophoretic mobility shift assay. Nuclear extracts were prepared from 1×10^6 treated cells using the NE-PER nuclear extraction reagents from Pierce Biotechnology. The nuclear factor- κ B (NF- κ B) consensus oligonucleotide (5'-AGT TGA GGC GAC TTT CCC AGG C-3') was purchased from Santa Cruz Biotechnologies, biotin end labeled, and used for the binding reactions; mutant oligos were used as control. Binding reactions and electrophoretic mobility shift assays (EMSAs) were performed using Light Shift chemiluminescent EMSA reagents from Pierce Biotechnology.

Incubation of cells with oligodeoxynucleotides. Cells were incubated with oligodeoxynucleotides to PKC- α and PKC- β along with high-glucose conditions with media change daily. The sequence for PKC- α isoenzyme-specific antisense oligonucleotide was 5'-CGC CGT GGA GTC GTT GCC CG-3'; the sense sequence was 5'-CGG GCA ACG ACT CCA CGG CG-3' (18). The PKC- β antisense oligonucleotide was 5'-CGC AGC CGG GTC AGC ATC-3'; the sense sequence was 5'-GAT GGC TGA CCC GGC TGC G-3' (18). All of the oligonucleotides were phosphorothioate modified and high-performance liquid chromatography purified. Following dose-response experiments for maximal effect, oligodeoxynucleotides were added to the cells at the concentration of 2 μ mol/l for PKC- α and 0.5 μ mol/l for PKC- β .

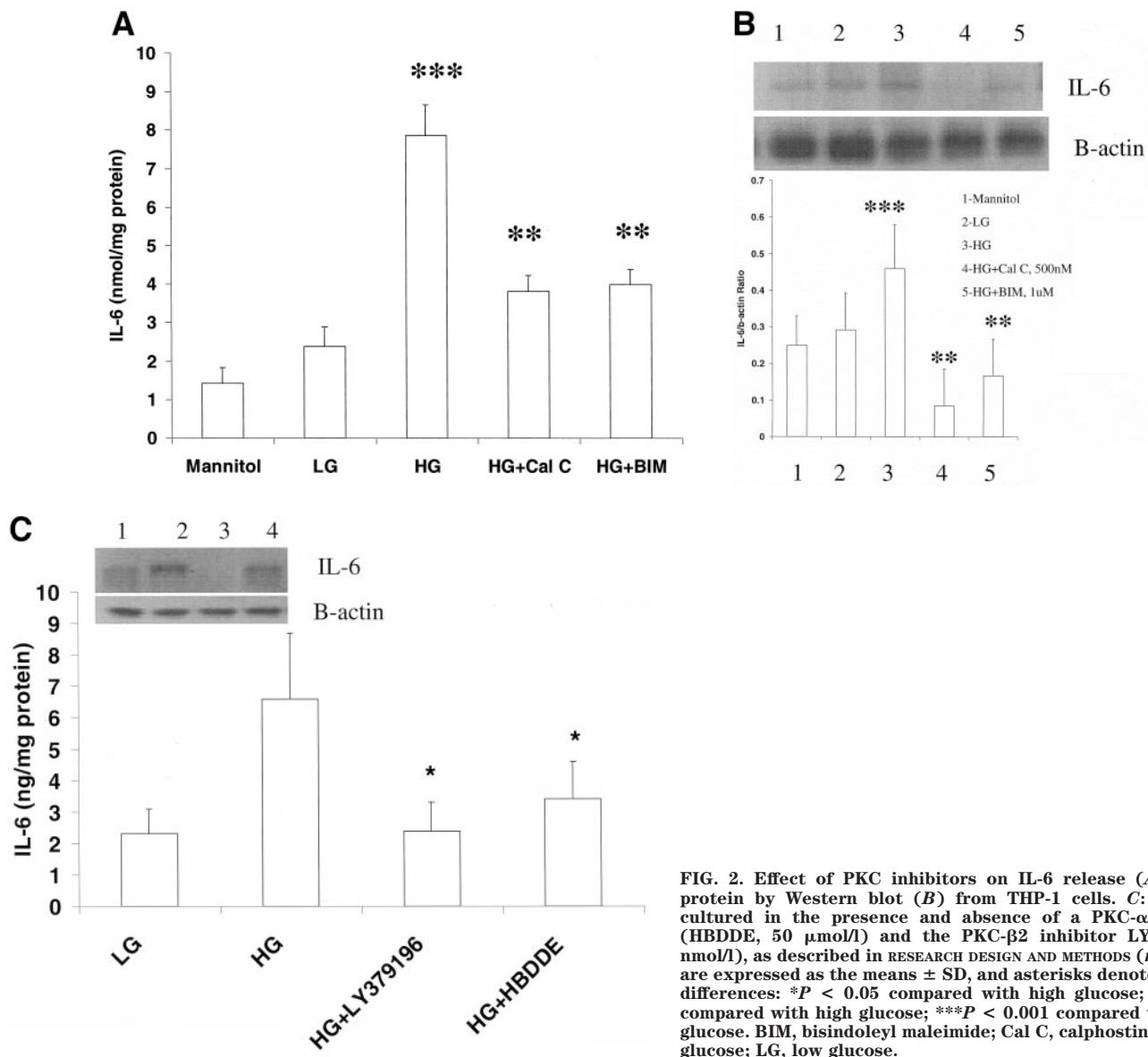


FIG. 2. Effect of PKC inhibitors on IL-6 release (A) and IL-6 protein by Western blot (B) from THP-1 cells. C: Cells were cultured in the presence and absence of a PKC- α/β inhibitor (HBDDE, 50 $\mu\text{mol/l}$) and the PKC- β inhibitor LY379196 (30 nmol/l), as described in RESEARCH DESIGN AND METHODS ($n = 4$). Data are expressed as the means \pm SD, and asterisks denote significant differences: * $P < 0.05$ compared with high glucose; ** $P < 0.01$ compared with high glucose; *** $P < 0.001$ compared with normal glucose. BIM, bisindoleyl maleimide; Cal C, calphostin C; HG, high glucose; LG, low glucose.

Treatment of cells with small interfering RNA. Small interfering RNA (siRNA) oligonucleotides to PKC isoforms were chemically synthesized using reagents from Ambion. siRNA construct software was used (Ambion), and the target sequences were: PKC- α , 5'-AAGCTCCATGTCACAGTACGA-3'; and PKC- β , 5'-AAGCGCTGCGTCATGAATGTT-3'. Both sense and antisense sequences were synthesized and then annealed. We mixed 20 $\mu\text{mol/l}$ of each sense and antisense oligonucleotide with 1 \times annealing buffer (6 mmol/l HEPES, pH 7.4; 20 mmol/l potassium acetate; and 400 $\mu\text{mol/l}$ magnesium acetate) followed by incubation at 90°C for 1 min and then at 37°C for 1 h. Then, the double-stranded siRNA was stored at -20°C in aliquots. Initial transfection experiments were performed with both siPORTamine and siPORTlipid and optimized with siPORTamine (Ambion, Austin, TX) using glyceraldehyde-3-phosphate dehydrogenase siRNA as control in THP-1 monocytes. Transfection efficiency was checked by RT-PCR, and >80% inhibition was observed. For further experiments with siRNAs to PKC- α and - β , cells ($1 \times 10^5/200 \mu\text{l}$) were plated on 24-well plates and incubated for 4 h with siPORTamine mixed with siRNA (10–50 nmol/l) and made up to a total volume of 250 μl . After 4 h, fresh media was added with or without high glucose (15 mmol/l) and incubated for an additional 20 h, and then it was collected for assay of PKC and IL-6 blotting and mRNA.

Statistical analysis. All experiments were performed on at least three occasions in duplicate or triplicate. Experimental results are presented as the means \pm SD. Paired t tests were used for data analysis, and significance was defined as $P < 0.05$.

RESULTS

Levels of IL-6, both secreted as well as intracellular IL-6, were significantly increased with high glucose (Fig. 1A and B, respectively). Furthermore, Western blotting revealed increased IL-6 protein in cell lysates with high glucose (Fig. 1C). Also, mRNA for IL-6 was increased with high glucose (Fig. 1D).

We have previously shown that superoxide release from monocytes under high glucose is mediated via activation of protein kinase C (PKC) (15). Thus, we first examined whether the increased superoxide anion released under high glucose could result in increased IL-6. Incubation of cells with PEG-SOD (50 units/ml) or MnTBAP (200 $\mu\text{mol/l}$) and DPI (5 $\mu\text{mol/l}$), while significantly decreasing superoxide anion release, failed to affect IL-6 release from monocytes under high glucose (high glucose: 5.2 ± 1.1 ng/mg protein; high glucose + PEG-SOD: 4.9 ± 1.4 ng/mg protein; high glucose + MnTBAP: 5.3 ± 1.2 ng/mg protein; high glucose + DPI: 4.8 ± 0.6 ng/mg protein; $n = 3$).

To ascertain the role of PKC, we examined the effect of

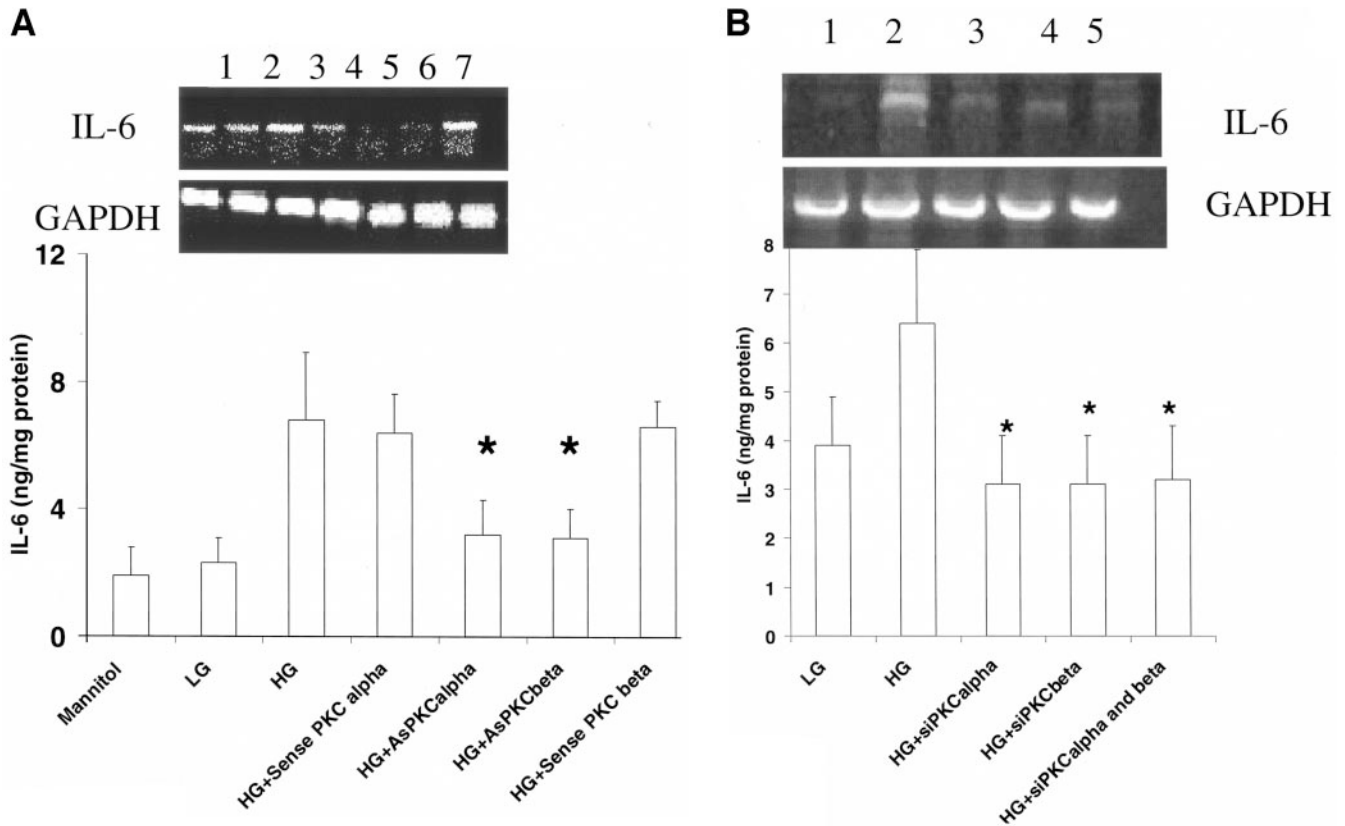


FIG. 3. Effect of antisense oligodeoxynucleotides (*A*) and siRNA to PKC- α and - β (*B*) on IL-6 release and IL-6 mRNA. Cells were cultured in the presence and absence of sense or antisense oligodeoxynucleotides to PKC- α and - β (*A*) or in the presence and absence of siRNA to PKC- α and/or - β , respectively (*B*), as described in RESEARCH DESIGN AND METHODS ($n = 4$). Data are the means \pm SD. * $P < 0.05$ compared with high glucose. AsPKCalpha, antisense oligodeoxynucleotides to PKC- α ; AsPKCbeta, antisense oligodeoxynucleotides to PKC- β ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HG, high glucose; LG, low glucose; siPKCalpha, siRNA to PKC- α ; siPKCbeta, siRNA to PKC- β .

PKC inhibitors on IL-6 release from THP-1 cells under high glucose. As shown in Fig. 2*A* and *B*, general PKC inhibitors calphostin C and bisindoleyl maleimide significantly decreased IL-6 release from monocytes under high glucose. To further examine which isoform of PKC mediates IL-6 release from human monocytes under high glucose, we examined the expression of the different isoforms. PKC- α and - β isoforms were increased with high glucose but not the PKC- δ , - γ , - θ , - ϵ , and - ξ isoforms (data not shown). Furthermore, the PKC- α/β inhibitor HBDDE as well as the specific PKC- β 2 inhibitor LY379196 decreased IL-6 release induced with high glucose (47 and 77%, respectively) (Fig. 2*C*). Thus, antisense oligodeoxynucleotides to PKC- α and - β isoforms were tested. Both antisense oligos significantly decreased high-glucose-induced IL-6 release (Fig. 3*A*). To further clarify the role of PKC- α and - β , we used siRNA technology to confirm which isoform is involved. As shown in Fig. 3*B*, both siRNA to PKC- α as well as siRNA to PKC- β significantly decreased IL-6 release from monocytes under high glucose (IL-6 levels for normal glucose: 3.9 ± 0.9 ng/mg protein; for high glucose: 6.4 ± 0.5 ng/mg protein; for high glucose + siRNA PKC- α : 3.1 ± 0.7 ng/mg protein; for high glucose + siRNA PKC- β : 3.1 ± 0.9 ng/mg protein; $n = 6$). Furthermore, the addition of siRNA to PKC- α and - β did not result in greater inhibition of IL-6 levels than either alone (IL-6: 3.4 ± 0.9 ng/mg protein)

We then explored the effect of the mitogen-activated

protein kinase (MAPK) pathway because PKC can activate MAPK. A specific inhibitor of p38MAPK, SB 202190 (10 $\mu\text{mol/l}$), but neither the extracellular signal-regulated kinase (ERK) inhibitor PD98059 (5 $\mu\text{mol/l}$) nor the JNK inhibitor LJNKI1 (5 $\mu\text{mol/l}$) significantly decreased IL-6 release from THP-1 cells under high glucose (Fig. 4*A*). Furthermore, incubation of THP-1 cells with PKC inhibitors significantly decreased p38MAPK, as assessed by Western blots (Fig. 4*B*).

Because the upregulation of IL-6 appears to be at the mRNA level, and the IL-6 promoter has NF- κ B response elements, we examined the effect of parthenolide (10 $\mu\text{mol/l}$) and Bay 11-7082 (5 $\mu\text{mol/l}$), both inhibitors of NF- κ B transcriptional activity on IL-6 message and protein. Both inhibitors significantly decreased IL-6 message and protein (Fig. 5*A* and *B*). Similar findings were obtained with two other NF- κ B inhibitors, CAPE (caffeic acid phenethyl ester) and SN-50 (synthetic peptide inhibitor of NF- κ B). Antisense oligos as well as siRNA to PKC- α and - β significantly decreased NF- κ B binding activity as analyzed by EMSA as well as I κ B kinase levels in the cytosol (Fig. 6*A* and *B*). Also, the p38MAPK inhibitor decreased NF- κ B binding activity.

Thus, IL-6 release from human monocytes under hyperglycemia appears to be mediated via upregulation of PKC- α/β , p38MAPK, and NF- κ B, resulting in increased mRNA and protein for IL-6. Because we have previously

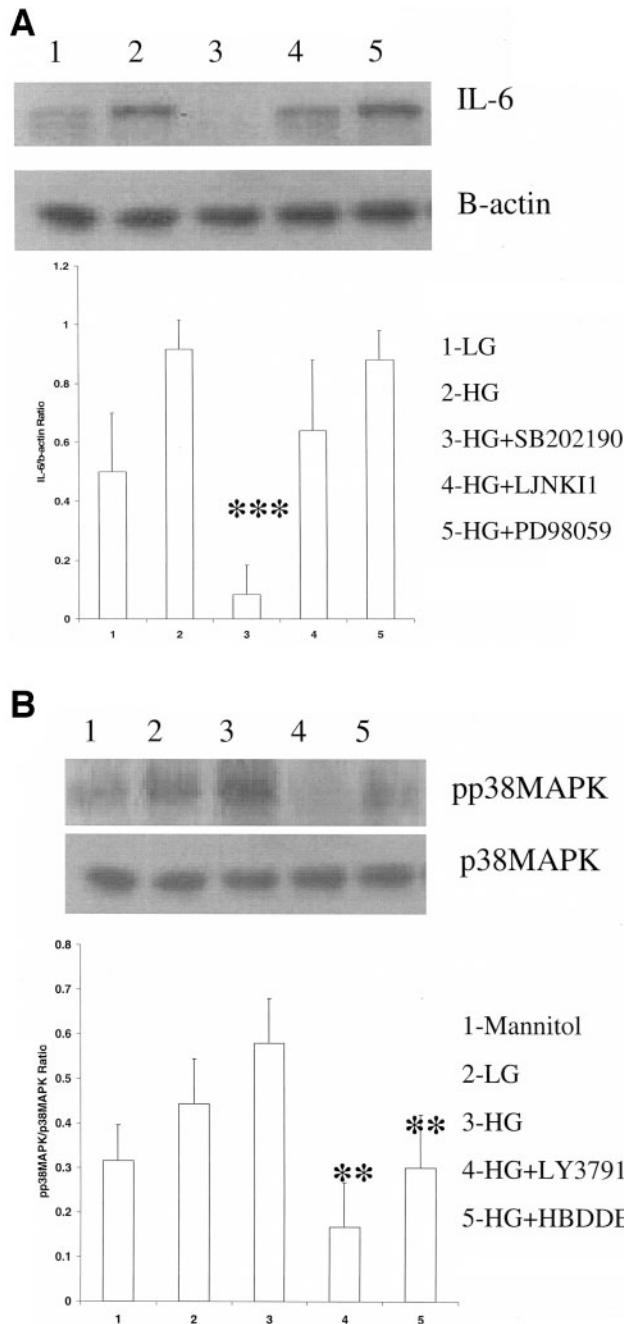


FIG. 4. A: Effect of MAPK inhibitors on IL-6 release from THP-1 cells. Cells were cultured in the presence and absence of inhibitors of p38MAPK, JNK, and ERK, and IL-6 Western blots were performed using β -actin as loading control, as described in RESEARCH DESIGN AND METHODS ($n = 4$). **B:** Effect of PKC inhibitors on p38MAPK on THP-1 cells. Cells were cultured in presence and absence of inhibitors of PKC, and pp38MAPK Western blots were performed using p38MAPK as loading control as described in RESEARCH DESIGN AND METHODS ($n = 4$). ** $P < 0.01$ compared with high glucose; *** $P < 0.001$ compared with high glucose. HG, high glucose; LG, low glucose.

shown that RRR- α tocopherol inhibits both PKC- α and - β , we tested the effect of RRR- α tocopherol on high-glucose-induced IL-6 release. RRR- α tocopherol significantly inhibited high-glucose-induced IL-6 release (IL-6 release for normal glucose: 2.2 ± 0.6 ng/mg protein; for high glucose: 5.0 ± 1.2 ng/mg protein; for high glucose + AT $100 \mu\text{mol/l}$: 3.9 ± 1.0 ng/mg protein [$P < 0.05$ compared with high glucose]; $n = 4$).

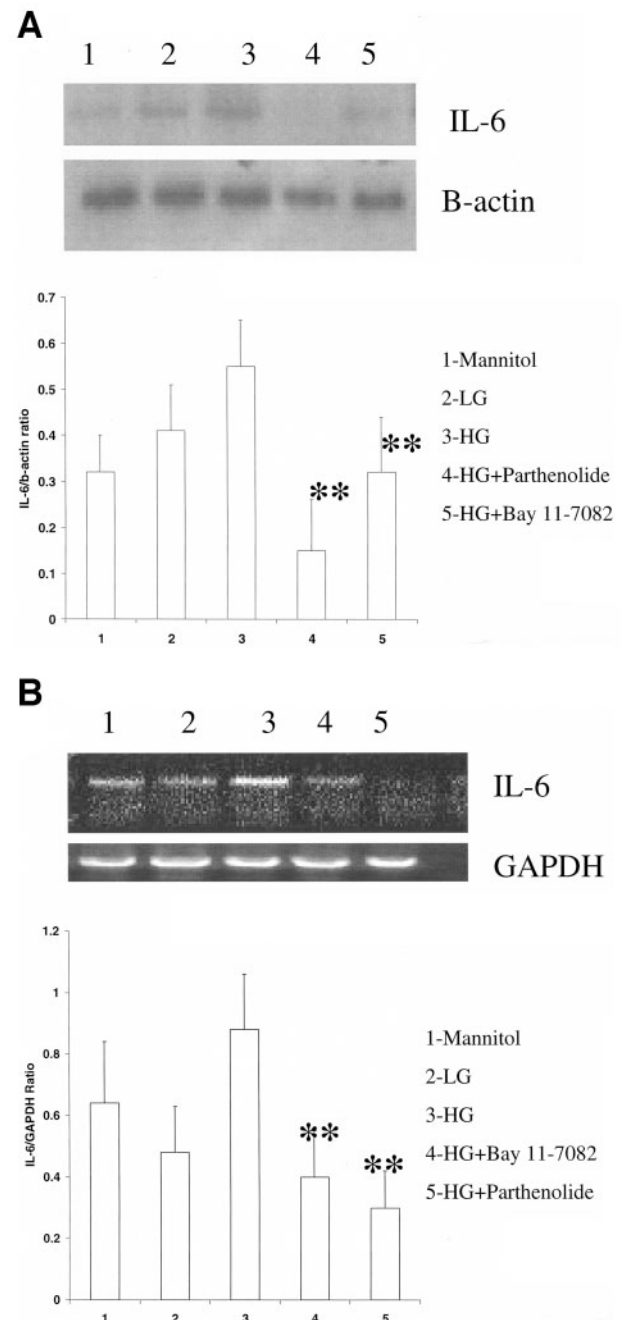


FIG. 5. Effect of NF- κ B inhibitors on IL-6 release from THP-1 cells. Cells were cultured in the presence and absence of inhibitors of NF- κ B and IL-6. Western blots (A) and IL-6 mRNA (B) were quantified using β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control, respectively, as described in RESEARCH DESIGN AND METHODS ($n = 3$). ** $P < 0.01$ compared with high glucose. HG, high glucose; LG, low glucose.

DISCUSSION

We previously showed that monocytes from type 2 diabetic patients released increased IL-6 compared with those from matched control subjects. Thus, we examined the potential mechanisms for the increased IL-6 release from monocytes under high glucose. We examined the following pathways: reactive oxygen species, PKC, MAPK, and NF- κ B, because all have been incriminated in diabetic vasculopathies (19,20).

Diabetes and cells cultured under high glucose are

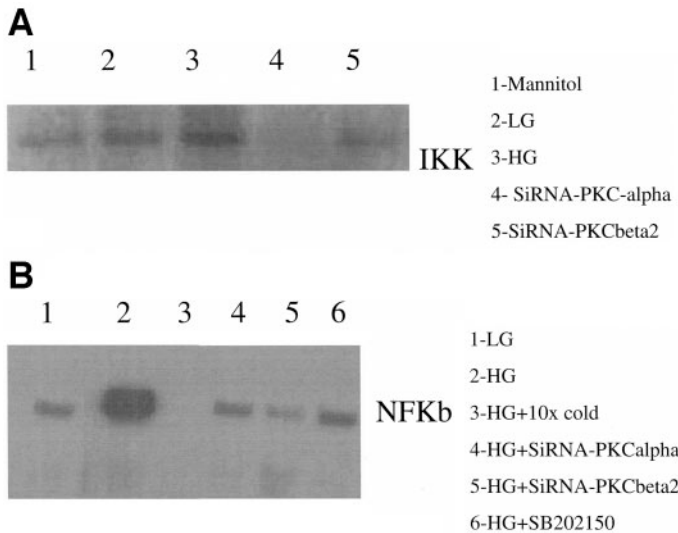


FIG. 6. Effect of siRNA on PKC- α and - β on p-IKK (A) and NF- κ B (B) in THP-1 cells. Cells were cultured in presence and absence of siRNA to PKC- α and - β , and p38MAPK inhibitor and Western blots for I κ B kinase (IKK) and EMSA for NF- κ B (NFKb) were performed as described in RESEARCH DESIGN AND METHODS ($n = 3$). HG, high glucose; LG, low glucose.

associated with increases in PKC activity (21). We have previously shown that superoxide release from monocytes under high glucose is mediated via activation of PKC- α (18). Reactive oxygen species inhibitors failed to affect IL-6 release from monocytes under high glucose. Thus, we examined the effect of PKC inhibitors on IL-6 release from THP-1 cells under high glucose. PKC inhibitors significantly decreased IL-6 release from monocytes under high glucose. Because different PKC isoforms are activated in different tissues, we examined activation of different PKC isoforms under high glucose. Only the PKC- α and - β isoforms were increased with high glucose in monocytes. Furthermore, the PKC- α/β inhibitor HBDDE as well as the specific PKC- β 2 inhibitor decreased IL-6 release induced with hyperglycemia. Koya and King (22) have shown that inhibition of the PKC- β 2 isoform can normalize retinal flow and glomerular filtration rate in streptozotocin-induced diabetic rats in parallel with inhibition of PKC activity. We have shown that PKC- α mediates superoxide anion release from monocytes under high glucose. Thus, antisense oligodeoxynucleotides to PKC- α and - β isoforms were tested. Both antisense oligos significantly decreased hyperglycemia-induced IL-6 release. siRNA technology was used to confirm which isoform is involved. Both siRNA to PKC- α as well as siRNA to PKC- β significantly decreased IL-6 release from monocytes under high glucose. However, the combination of siRNA to PKC- α and - β was not additive, suggesting that both mediate their effects on IL-6 release from monocytes via common downstream pathways.

High glucose has been shown to activate p38MAPK in vascular smooth muscle cells (23,24). In addition, production of inflammatory cytokines such as TNF and IL-6 by activated rat smooth muscle cells was regulated by the p38MAPK pathway (25). Also, activation of PKC could directly activate the MAPK pathway. A specific inhibitor of p38MAPK, SB 202190, but neither the ERK inhibitor PD98059 nor the JNK inhibitor LJNKI1 significantly decreased IL-6 release from THP-1 cells under high glucose.

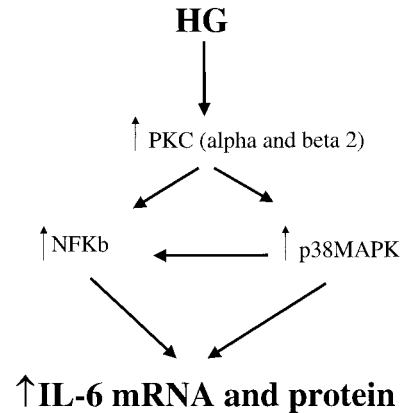


FIG. 7. Schema depicting the mechanisms by which hyperglycemia (HG) activates IL-6 mRNA and protein in THP-1 cells via activation of PKC, p38MAPK, and NF- κ B (NFKb).

Thus, in this study, we have shown that inhibition of p38MAPK significantly decreases IL-6 release from human monocytes.

Previous reports indicate that the NF- κ B binding site located between positions -72 and -63 on the IL-6 gene is important for the induction of IL-6 (26). p38MAPK has been shown to induce IL-6 expression in neonatal rat cardiomyocytes via activation of NF- κ B (27). Because the upregulation of IL-6 appears to be at the mRNA level, and the IL-6 promoter has NF- κ B response elements, we examined the effect of parthenolide and Bay 11-7082, both inhibitors of NF- κ B transcriptional activity, on IL-6 message and protein. Both inhibitors significantly decreased IL-6 message and protein. High glucose has also been shown to induce TNF via the following: oxidant stress, p38MAPK activity in monocytes (28), and increased NF- κ B activity. Shanmugam et al. (29) recently demonstrated in a microarray of monocytic cells cultured under high glucose that there was a significant induction of IL-1, TNF, macrophage chemoattractant protein-1 (MCP-1), etc.; that MCP-1 is upregulated via increased NF- κ B transcription; and that p38MAPK and ERK inhibitors decreased MCP-1 release. Hence, we examined whether p38MAPK inhibitors also decrease NF- κ B activity. As shown by EMSA, siRNA to PKC- α/β as well as p38MAPK inhibitors significantly decreased high-glucose-induced NF- κ B binding and resulted in decreased IL-6 release from monocytes under high glucose.

Thus, we conclude that under high glucose, monocytes secrete increased amounts of IL-6 via upregulation of PKC- α and - β and p38MAPK and NF- κ B activity, leading to increased IL-6 transcription and release (Fig. 7).

ACKNOWLEDGMENTS

This investigation was supported by National Institutes of Health Grant K24 AT00596, the Juvenile Diabetes Foundation (to I.J.), and the American Diabetes Association Junior Faculty Award (to S.D.).

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