Hyperglycemia-Induced Activation of Nuclear Transcription Factor κB in Vascular Smooth Muscle Cells

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The transcriptional nuclear factor (NF)-κB can be activated by diverse stimuli such as cytokines, mitogens, oxidative stress, and lipids, leading to the transactivation of several genes that play important roles in the development of atherosclerosis. Because oxidative stress may play a key role in the pathogenesis of diabetic vascular disease, we have examined whether culture of porcine vascular smooth muscle cells (PVSMCs) under high glucose (HG) conditions (25 mmol/l) to simulate the diabetic state can lead to the activation of NF-κB, and also whether cytokine- or growth factor–induced NF-κB activation is altered by HG culture. We observed that PVSMCs cultured in HG showed significantly greater activation of NF-κB in the basal state compared with cells cultured in normal glucose (NG) (5.5 mmol/l). Treatment of the cells with cytokines, such as tumor necrosis factor (TNF)-α and interleukin-1β, or with growth factors, such as platelet-derived growth factor, insulin-like growth factor-I, and epidermal growth factor, all led to NF-κB activation in cells cultured in both NG and HG. However, their effects were markedly greater in HG. The augmented TNF-α–induced NF-κB activation in HG was associated with increased TNF-α–mediated transcriptional activation of the vascular cell adhesion molecule-1 promoter. Immunoblotting with an antibody to the p65 subunit of NF-κB indicated that the levels of this protein were higher in the nuclear extracts from cells cultured in HG compared with NG. Cells cultured in HG also produced significantly greater amounts of the reactive oxygen species superoxide. HG-induced NF-κB activation was inhibited by a protein kinase C inhibitor, calphostin C. These results suggest that hyperglycemia-induced activation of NF-κB in VSMCs may be a key mechanism for the accelerated vascular disease observed in diabetes. ***Diabetes*** **48:855–864, 1999**

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AGE, advanced glycation end product; ANOVA, analysis of variance; BSA, bovine serum albumin; CAT, chloramphenicol acetyl transferase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; HG, high glucose; ICAM, intercellular adhesion molecule; IL, interleukin; MCP, monocyte chemotactic protein; NF, nuclear factor; NG, normal glucose; NIH, National Institutes of Health; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PVSMC, porcine vascular smooth muscle cell; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule; VSMC, vascular smooth muscle cell.

The transcriptional nuclear factor (NF)-κB is widely distributed in several cell types. It mainly consists of a dimer of the two subunits p50 and p65 (Rel A), and in its inactive state it is sequestered in the cytoplasm with an inhibitor protein called IκB (1,2). Activation of NF-κB leads to the release of the inhibitory IκB subunit from the heterotrimeric complex followed by translocation of the dimer to the nucleus, where it initiates transcription (2). NF-κB activity can be induced in a variety of cells in response to treatment with various agents such as cytokines, oxidative stress, and oxidized LDL (3,4), and it can control the transcription of several genes involved in immune and inflammatory responses, growth, and adhesion (4,5).

Increasing evidence suggests that NF-κB/Rel transcription factors may play important roles in atherosclerosis (6–9). NF-κB has been shown to regulate a variety of genes that are induced in the atherosclerotic lesion, including genes encoding tumor necrosis factor (TNF)-α (6,10), interleukin (IL)-1β (11), vascular cell adhesion molecule (VCAM)-1 (12,13), and intercellular adhesion molecule (ICAM)-1 (14).

Prolonged hyperglycemia is believed to be one of the major causes of vascular complications associated with diabetes (15). However, the mechanisms whereby hyperglycemia alters the behavior of vascular cells are not clearly understood. Persistent hyperglycemia in vivo and in vitro can lead to the activation of protein kinase C (PKC), which has been associated with diabetic complications (16). Hyperglycemia can also lead to the accumulation of glycated proteins called advanced glycation end products (AGEs) (17). These AGEs, acting through their specific receptors on vascular cells, can lead to oxidant stress and cellular dysfunction in the pathology of atherosclerosis as well as contribute to the development of diabetic complications (17–20). Elucidation of the mechanisms that regulate vascular smooth muscle cell (VSMC) migration and proliferation is crucial to understanding the increased risk of cardiovascular disease in diabetes. We have previously shown that porcine vascular smooth muscle cells (PVSMCs) cultured under high glucose (HG) conditions (25 mmol/l) to simulate the diabetic state proliferate at a significantly faster rate than cells cultured under normal glucose (NG) conditions (5.5 mmol/l) (21). In addition, we have also shown that the growth-promoting effects of angiotensin II and the chemotactic effects of platelet-derived

growth factor (PDGF) are enhanced in VSMCs cultured under hyperglycemic conditions (22,23). Evidence suggests that VSMCs express a constitutive NF-κB–like activity (24) that is essential for proliferation (25). Furthermore, Bourcier et al. (26) have recently suggested that this constitutive activity could arise from serum components.

The function of NF-κB in VSMCs as well as its role in diabetic vascular disease have not been well studied. Because NF-κB is a pleiotropic oxidant-sensitive transcriptional factor and hyperglycemia-induced oxidative stress may play a key role in the pathogenesis of diabetic vascular disease, in the present study, we have examined our hypothesis that hyperglycemia itself can lead to the activation of NF-κB in PVSMCs. Furthermore, we have also examined whether certain factors implicated in atherogenesis, such as specific growth factors and cytokines, can lead to the activation of NF-κB in PVSMCs and whether their effects are potentiated by hyperglycemia.

RESEARCH DESIGN AND METHODS

Cell culture. Primary cultures of PVSMCs were obtained as described earlier (21,27). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing NG (5.5 mmol/l) and 10% fetal calf serum (FCS). For studies under hyperglycemic conditions, cells were grown in DMEM with HG (25 mmol/l) and 10% FCS for one or more passages. All experiments were done in one to four passages, except in experiments examining short-term effects of HG, in which HG was added for 2–8 days.

Incubation of cells with cytokines, growth factors, and other agents. VSMCs (80–90% confluent) growing in 100-mm dishes in NG or HG growth medium were made quiescent by placement in serum depletion medium—DMEM with 0.2% bovine serum albumin (BSA) and 0.4% FCS—for 24 h. Cells were washed with phosphate-buffered saline (PBS) and preincubated for 30 min in depletion medium containing 0.2% BSA only. Agents were then added and incubated for 3 h. Cytokines, such as TNF-α and IL-1β (R&D Systems, Minneapolis, MN), and growth factors, such as epidermal growth factor (EGF), PDGF-BB (Gibco, Gaithersburg, MD), and IGF-I (Mallinckrodt Chemical, Paris, KY), were diluted in sterile PBS and added to the cells at a concentration of 5–10 ng/ml. Cells were processed for nuclear and cytosolic protein extraction at the end of the incubation period as described below.

Preparation of nuclear and cytosolic extracts. Nuclear extracts were prepared according to Marui et al. (28). Briefly, after the incubations, cells from 100-mm dishes were washed twice with ice-cold PBS, scraped into 1 ml PBS, and spun down at 3,500 rpm for 4 min at 4°C. Cell pellets were resuspended in wash buffer containing 10 mmol/l HEPES, pH 7.9, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml antipain and leupeptin, and 2.4 μg/ml aprotinin and then lysed in lysis buffer containing wash buffer with 0.1% NP40 by incubating on ice for 10 min. Lysates were then centrifuged at 3,500 rpm for 4 min. Supernates were removed and stored at –70°C as cytosolic fractions. Nuclear pellets were washed with wash buffer and resuspended in buffer containing 20 mmol/l HEPES, pH 7.9, 25% glycerol, 420 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, and 0.2 mmol/l EDTA, with the protease inhibitors at the same concentrations used in the wash buffer. This suspension was incubated for 30 min at 4°C followed by centrifugation at 10,000*g*. The resulting supernatant containing the nuclear proteins was stored at –70°C until use.

Electrophoretic mobility shift assay. Oligonucleotides corresponding to κB-like sites (underlined) in the human VCAM-1 promoter region (13) 5′-CTGC CCTGGGTTTCCCCTTGAAGGGATTTCCCTCCGCCT-3′ were synthesized in the City of Hope National Medical Center's DNA synthesis facility. Oligonucleotides with binding sites for AP-2 or Sp1 (controls) were obtained from Promega (Madison, WI). After annealing, double-stranded DNA was labeled with [γ-^{32}P]ATPusing T4 kinase (Stratagene, La Jolla, CA) and purified on Sephadex G-25 column. Nuclear protein (5–10 μg) was incubated with ^{32}P-labeled oligonucleotide probe (20,000 cpm) at room temperature for 20 min in binding buffer containing 12 mmol/l HEPES (pH 7.9), 4 mmol/l Tris-HCl (pH 7.9), 60 mmol/l KCl, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 1 mmol/l PMSF, 12% glycerol, 5 μg of BSA, 2 μg of poly deoxyinosinic deoxycytidylic acid. Protein-DNA complexes were resolved on 4% native polyacrylamide gels using 1× Tris-glycine buffer (pH 8.5). For the supershift analyses, 2 μg each of the antibodies to the NF-κB subunit proteins (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with the nuclear extracts and the labeled probe for 1 h at 4°C. Then the gel shift analyses were carried out as usual. Gels were dried and visualization and quantitation of radioactive bands were carried out on a PhosphorImager (Molecular Dynamics, San Jose, CA) using Imagequant software (National Institutes of Health, Bethesda, MD).

Competition experiments were performed by 10-min preincubation of nuclear protein with 40 molar excess of unlabeled wild-type VCAM oligonucleotide or mutant VCAM oligonucleotide (5′-CTGCCCTG**A**GT**CA**C**G**C CTTGAAG**A**GA**CA**TC**A**CTCCGCCT-3′: four base mutations in each NF-κB binding site, indicated in bold). Electrophoretic mobility shift assays (EMSAs) were then performed as described above.

Immunoblotting to detect the p50 and p65 subunits of NF-κB. To determine the cellular levels of p50 or p65 protein subunits of NF-κB, nuclear or cytosolic protein extracts (5–10 μg for p65 and 20–30 μg for p50) were resolved on 8% SDS polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes. After blocking nonspecific sites, the blots were then incubated overnight with a rabbit polyclonal antibody to the p50 or p65 subunit of NF-κB (1:1,000). Washed blots were then incubated with an alkaline phosphatase-labeled second antibody (Tropix, Bedford, MA) at 1:30,000 dilution and visualized by chemiluminescence using the Tropix detection system. Bands were quantitated on a computerized densitometer (SCISCAN 5000; United States Biochemical, Cleveland, OH). Equal loading of protein was confirmed by staining with Coomassie Blue.

Transient DNA transfections and chloramphenicol acetyl transferase assays. Transient DNA transfections and chloramphenicol acetyl transferase (CAT) assays were carried out as described earlier (28,29). Briefly, PVSMCs that had been through one passage in HG were split 24 h before transfections to attain 60% confluence in 100-mm culture dishes. Cells were placed in medium containing 10% FCS and transfected with 10 μg reporter plasmid p85VCAMCAT or pSV_2CAT (positive control) (13) by the calcium phosphate coprecipitation technique for 8 h using standard techniques (29–31). After a 12-h recovery period in fresh DMEM (NG or HG) containing 10% FCS, cells were treated with or without 5 ng/ml TNF-α. At 16–18 h later, cell extracts were prepared by rapid freeze-thaw in 0.25 mol/l Tris, pH 8.0, and protein content was determined. Protein (40 μg) from each cell extract was assayed for CAT activity by standard protocols (29,30). p85VCAMCAT and the positive control pSV_2CAT plasmid were generous gifts from Dr. Douglas Dean (Washington University, St. Louis, MO).

Measurement of superoxide production by the VSMCs. Cells growing in DMEM NG (5.5 mmol/l) were placed in growth medium containing 12.5 mmol/l or 25 mmol/l (HG) for 1 week. Confluent cells were then made quiescent by placement in DMEM (appropriate glucose) + 0.2% BSA + 0.4% FCS for 24 h. Cells were then preincubated for 30 min in fresh depletion medium containing 0.2% BSA only, and then they were treated for 4 h with or without TNF-α (5 ng/ml). Cells were then processed for superoxide measurement by the lucigenin chemiluminescence method as described by Griendling et al. (32). Cells were detached with type I collagenase (1 mg/ml) (32) and cell number adjusted to 1 million/ml. Dark adjusted scintillation vials containing 1.25 ml of oxygenated Krebs-HEPES buffer with 0.25 ml lucigenin (0.125 mmol/l) were placed in a Beckman LS-6500 model (Fullerton, CA) liquid scintillation counter, and the background counts were recorded in the out-of-coincidence mode for 5 min. Cells (0.5×10^6) were kept at room temperature for 5 min and added to the corresponding vials, and the counts were recorded continuously for 5 min.

Data analyses. Results are expressed as means ± SE. Statistical significance was assessed with Student's *t* tests or one-way analysis of variance (ANOVA) using Prism software (Graphpad, San Diego, CA). Tukey-Kramer posttests were performed when appropriate.

RESULTS

Effects of high glucose culture on NF-κB activity in VSMCs. To determine whether HG culture can alter basal NF-κB activity in VSMCs, we examined NF-κB activity in VSMCs that had been cultured for at least one passage in HG (25 mmol/l) medium. Cells growing in NG (5.5 mmol/l) or HG medium were made quiescent for 24 h and then treated for 3 h with or without TNF-α (5 ng/ml). Figure 1*A* shows the EMSA and depicts the binding of equal amounts of nuclear protein extracts from these cells to an oligonucleotide containing two NF-κB motifs derived from the human VCAM-1 promoter. It is seen that basal NF-κB activity under unstimulated conditions in HG (*third lane from the left*) was greater than that in the cells cultured in NG (*lane 1*). Treatment with TNF-α led to a marked increase in NF-κB activity in PVSMCs cultured in both NG and HG (Fig. 1*A*). However, it is seen that the effect of TNF-α was distinctly greater in HG. Figure 1*B* shows a bar graph of the PhosphorImager quantitation of the results of several experiments and reveals a significant 1.7-fold

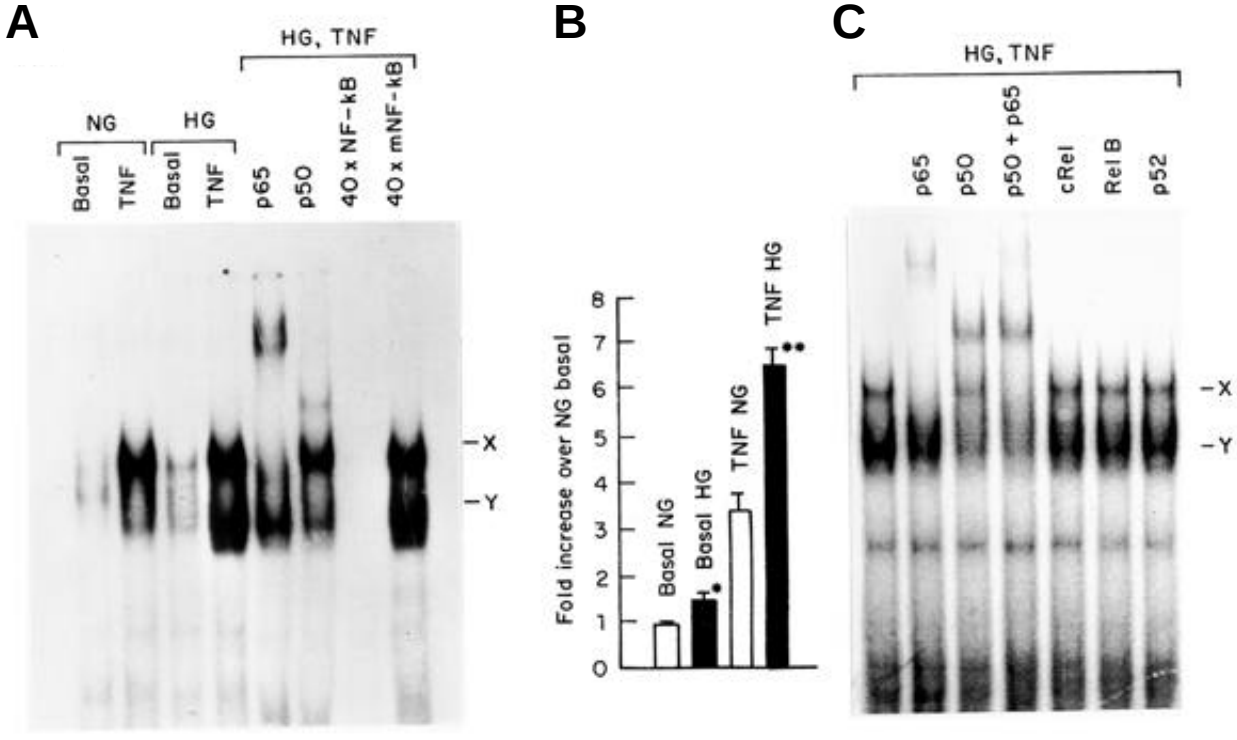

FIG. 1. Hyperglycemia-induced modulation of NF-κB DNA binding activity in VSMCs. *A*: Serum-starved VSMCs growing for two passages in NG or HG media were treated for 3 h alone or with 5 ng/ml of TNF-α. Nuclear proteins were prepared and subjected to EMSA to determine activation of NF-κB using an NF-κB consensus VCAM oligonucleotide as described under METHODS. For the competition studies shown in the *last two lanes*, nuclear extracts (5 µg each) (from TNF-α–treated HG cells) were pretreated with either 40 × excess cold wild-type VCAM promoter sequence oligonuleotide or 40 × excess cold mutant VCAM oligonucleotide. These samples were then subjected to DNA binding reactions with the labeled VCAM oligonucleotide and EMSA. Results demonstrate specificity of the binding. For the supershift experiments with p65 and p50 antibodies (*lanes 5* and *6*), nuclear extracts from TNF-α–treated cells in HG were incubated with the respective antibodies for 1 h at 4°C and then EMSAs were run as usual. Specific complexes X and Y are indicated. Similar results were obtained in at least four independent experiments. *B*: Bar graph showing the means ± SE of results from the PhosphorImager quantitation of the EMSA results obtained from six experiments. **P* < 0.005 vs. NG basal, ***P* < 0.01 vs. NG TNF-α by ANOVA using Prism software (GraphPad). *C*: Supershifting with antibodies to NF-κB subunits p65, p50, cRel, RelB, and p52/49. Nuclear extracts from TNF-α–stimulated cells in HG were preincubated with the respective antibodies and then EMSAs were run.

increase in basal activity in HG relative to NG ($P < 0.001$) and a 2-fold increase in TNF-α effects in HG ($P < 0.001$). These results suggest that basal NF-κB activity as well as the effects of TNF-α were enhanced under hyperglycemic conditions.

Mainly two bands of specific DNA binding complexes, designated X and Y, were seen, particularly under TNF-α–stimulated conditions. The identity of the subunit proteins bound to the NF-κB probe was demonstrated by supershifting with antisera to the Rel family members p50 and p65, which have recently been shown to be the key Rel proteins expressed in VSMCs (26), and with antisera to other NF-κB subunit members: cRel, RelB, and p52/49. Nuclear extracts from HG cells treated with TNF-α were used for these purposes. Figure 1*A* (*lane 5*) shows that an antibody to p65 (RelA) retarded the upper slower moving band (X), leaving most of the lower faster migrating band Y unaffected. *Lane 6* shows that an antibody to p50 interacted and retarded the complexes in the lower band Y and also partly affected the upper band X (Fig. 1*A*). Similar results are clearly evident in *lanes 2* and *3* of Fig. 1*C*, which also shows that the addition of both the p65 and p50 antibodies completely abrogated all binding (*lane 4*). Furthermore, Fig. 1*C* shows that antibodies to the other Rel subunits (cRel, RelB, and p52) did not induce any supershifting. These results suggest that most of the binding activity arose from p50/p65 dimers. This is similar to results in VSMCs obtained recently by other researchers (26).

Competition experiments were performed to confirm the specificity of the protein DNA complexes for NF-κB sequence. Figure 1*A* (*last two lanes*) shows a representative experiment. *Lane 7* had nuclear extract from TNF-α–treated HG cells that was incubated with ^{32}P-labeled wild-type VCAM oligonucleotide as well as 40 × excess cold wild-type VCAM oligonucleotide. This led to specific blocking of all DNA binding. In contrast, the last lane (*lane 8*) shows that addition of 40 × cold mutant VCAM oligonucleotide failed to block the DNA binding in both complexes X and Y, which thus represents specific NF-κB–like DNA binding activity in response to TNF-α.

To confirm specificity of the observed effects, we also examined binding to oligonucleotides containing DNA binding elements for two other transcription factors, namely, Sp1 and AP-2. Results in Fig. 2 show that the regulation pattern is completely different from that seen with NF-κB. Thus Sp1 was not regulated significantly by either HG or TNF-α. In the case of AP-2, HG had no effect relative to NG in the basal state, while TNF-α in fact had a negative effect on AP-2

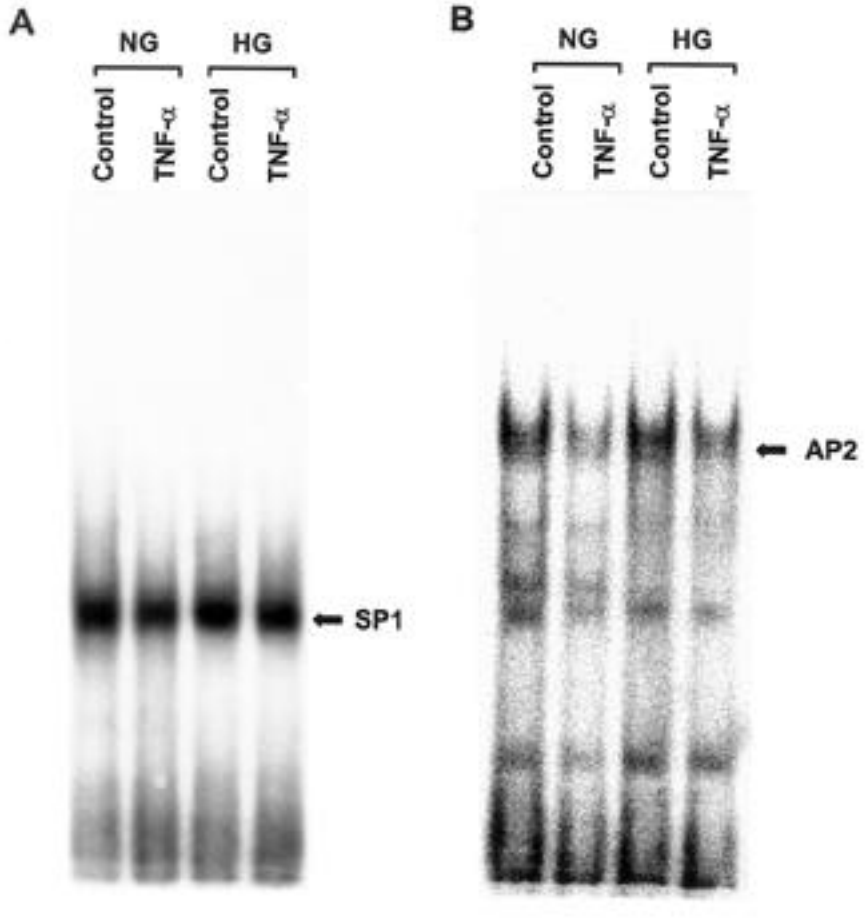

FIG. 2. Binding of nuclear extracts to oligonucleotides containing binding sites for the transcriptional factors Sp1 or AP-2. VSMCs in NG or HG were treated for 3 h with or without TNF-α (5 ng/ml). Nuclear proteins were incubated with labeled oligonucleotides containing binding sites for Sp1 or AP-2 and then EMSAs were run. *A*: DNA binding pattern for Sp1. *B*: DNA binding pattern for AP-2.

binding. These results indicate that the results observed in Fig. 1 are specific for NF-κB.

We also examined whether glucose concentrations in the range of 10–15 mmol/l would induce NF-κB activation similar to the 25 mmol/l used in Fig. 1 and other experiments. For this, cells were cultured in medium containing 5.5, 10, 12.5, or 15 mmol/l glucose. Figure 3 shows that, even at 10 mmol/l glucose, there was a distinct upregulation of NF-κB activity both in the unstimulated and in the TNF-α–treated cells. Furthermore, these effects were sustained in the cells cultured in 12.5 and 15 mmol/l elevated glucose concentrations. Thus, increased NF-κB activation is evident at glucose concentrations that are likely to be commonly present in diabetic patients.

In other experiments, we examined basal and TNF-α–induced NF-κB activity in cells that were treated with 25 mmol/l HG for various time periods (2–8 days). Figure 4 shows that increased NF-κB activity was observed in unstimulated as well as in cytokine-treated cells even as early as 2 days in HG. This increase elicited by HG remained sustained for up to 8 days (Fig. 4) and also for up to four passages or 30–40 days in HG, as seen in Fig. 1 and the ensuing Fig. 5.

We also compared the effects of another cytokine, IL-1β, which has been shown to induce VSMC proliferation and may play a role in atherosclerosis. We observed that while IL-1β activated NF-κB only weakly in NG (1.7 ± 0.3-fold over control), its effects were much greater (2.8 ± 0.4-fold) under HG conditions (results not shown). Thus the effects of both these cytokines, TNF-α and IL-1β, on NF-κB activation were enhanced under HG conditions.

Hyperglycemia augments growth factor–induced activation of NF-κB in PVSMCs. Growth factors such as PDGF and IGF-I have been shown to play important roles in VSMC proliferation and migration and hence in the development of the atherosclerotic lesion. We tested whether some of these growth factors can induce NF-κB activation in PVSMCs and further whether their effects were augmented under HG conditions. Representative EMSA results are seen in Fig. 5. Figure 5*A* shows the effects of PDGF, and Fig. 5*B* shows the effects of IGF-I and EGF added at a concentration of 10 ng/ml each for a period of 3 h to PVSMCs cultured in NG or in HG. Figure 5*A* and *B* shows that basal NF-κB was clearly higher (1.8 ± 0.2-fold, $P < 0.001$ vs. NG) in HG controls compared with NG controls, as seen in the previous experiments. Further, Fig. 5*A* shows that PDGF led to a greater increase in NF-κB activation in HG (2.9 ± 0.4-fold, $P < 0.01$ vs. control) than in NG (1.9 ± 0.2-fold, $P < 0.01$ vs. control). EGF activated NF-κB feebly in cells grown under NG conditions (Fig. 5*B*, *lane 2*) (1.4 ± 0.3-fold, $P < 0.03$ vs. control). In contrast, activation of NF-κB by EGF in cells grown in HG (*lane 5*) was markedly greater (3.0 ± 0.4-fold, $P < 0.001$ vs. control). IGF-I treatment also led to greater NF-κB activation in HG compared with NG (2.7 ± 0.3-fold vs. 1.8 ± 0.1-fold, $P < 0.01$ vs. each control, Fig. 5*B*). Thus, the effects of these key growth factors on NF-κB activation in VSMCs were also enhanced under HG conditions.

Regulation of the p50 and p65 subunits of NF-κB by high glucose, TNF-α, or growth factors. p65 is the active subunit of the NF-κB complex, and it has been shown to play an important role in VSMC adherence and proliferation

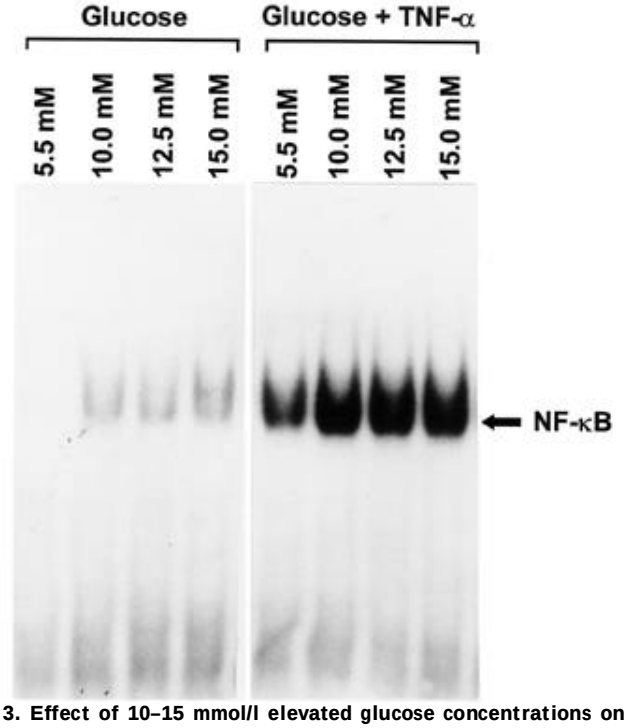

FIG. 3. Effect of 10–15 mmol/l elevated glucose concentrations on NF-κB activity in VSMCs. PVSMCs were cultured for one passage (8 days) in medium containing either 5.5 mmol/l glucose (NG) or increased glucose concentrations of 10, 12.5, or 15 mmol/l. They were then serum-starved for 24 h in the medium containing the respective glucose concentrations followed by a 3-h treatment with or without TNF-α (5 ng/ml).

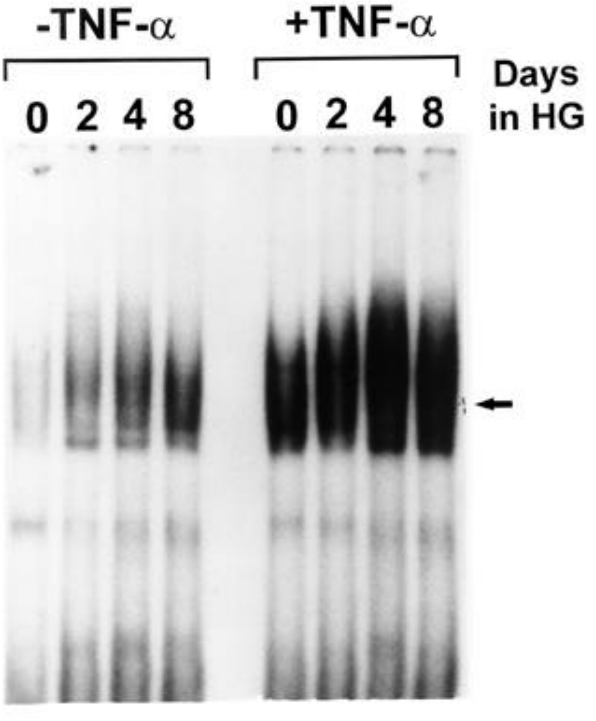

FIG. 4. Time course of HG effects on NF-κB activity in PVSMCs. PVSMCs growing under NG conditions were treated for various short time periods (2, 4, and 8 days) with HG (25 mmol/l). Cells were then serum-starved for 24 h and treated with or without TNF-α for 3 h. NF-κB activities were assessed by EMSA.

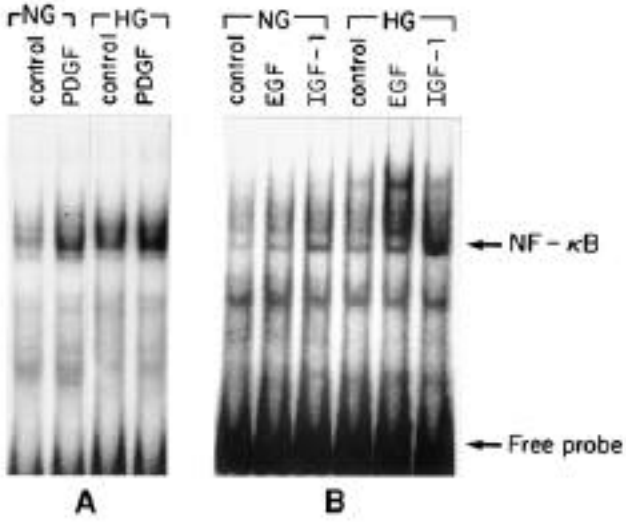

FIG. 5. Growth factors show increased activation of NF-κB under hyperglycemic conditions. Serum-starved PVSMCs growing under NG or HG conditions were treated for 3 h without or with one of the following growth factors: PDGF (*A*), IGF-I (*B*), or EGF (*B*) at 10 ng/ml concentration. EMSAs were performed on the nuclear proteins. Results shown are representative of three experiments. As noted in the RESULTS, significant increases in NF-κB activity were induced by all three growth factors and were greater in HG.

(33). Binding of p65 to DNA therefore transactivates various genes whose expressions are important in VSMC pathobiology. To evaluate expression of p65, we used immunoblotting with a specific antibody. Figure 6*A* shows a representative immunoblot depicting the expression of p65 protein in nuclear extracts prepared from cells cultured in NG versus HG in three separate dishes. Figure 6*B* shows the densitometric analysis of the bands obtained from several experiments. The results show that there was a significant twofold greater level of the p65 protein in the cells cultured in HG conditions compared with NG ($P < 0.005$).

To confirm that the observed results were not artifacts of cytosolic contamination in nuclear extracts, we probed both nuclear and cytosolic extracts from the same VSMCs (with and without TNF-α treatment) with p65 antibodies. Results seen in Fig. 6*C* again reveal higher basal p65 levels in HG nuclear extract versus NG. TNF-α treatment increased nuc-lear p65 levels in NG as well as HG. However, these levels were greater in HG nuclear extracts. In contrast, the cytosolic extracts depicted no such regulation, since no differences in cytosolic p65 levels were observed either in unstimulated cells or in those treated with TNF-α. This suggested that increased p65 translocation may be a potential mechanism for enhanced NF-κB activation observed in HG. This process did not result in an appreciable loss of cytosolic p65 content. It is likely that the levels of cytosolic p65 are not altered by either HG or the cytokine because only a small fraction of the total cytosolic pool of p65 is translocated to the nucleus upon activation. This is similar to observations by other researchers (26). Equal loading of protein was confirmed by Coomassie Blue staining.

Figure 7 shows p65 levels in nuclear protein extracts from PVSMCs grown under NG or HG conditions and stimulated for 3 h with or without TNF-α, PDGF, or EGF. Basal p65 lev-

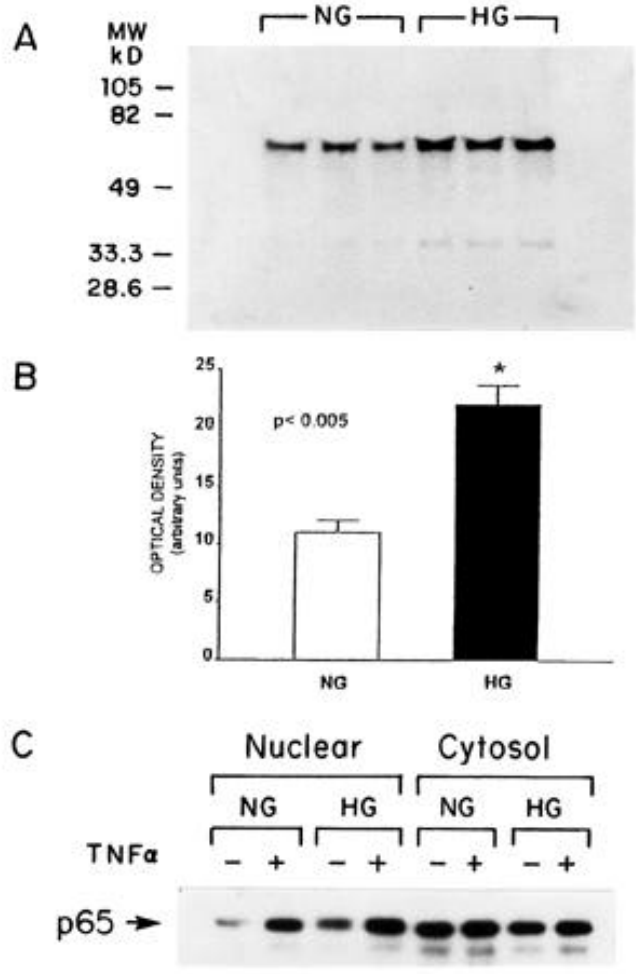

FIG. 6. Expression of p65 subunit of NF-κB in VSMCs cultured in NG versus HG. PVSMCs growing in NG or HG medium were serum-depleted for 24 h in DMEM (NG or HG) containing 0.2% BSA and 0.4% FCS. They were then placed in fresh medium containing 0.2% BSA only and incubated for 3 h with or without TNF-α. Nuclear and cytosolic extracts from these cells (10 µg/sample) were subjected to Western immunoblotting to detect p65 levels using a specific antibody (1:1,000). *A*: Representative immunoblot with three separate samples, each from unstimulated NG and HG cells, depicting the 65-kDa band of the p65 protein subunit. *B*: Bar graph showing the densitometric quantitation of the bands obtained from six experiments. Results are means ± SE, $P < 0.005$ vs. HG by Student's t test. *C*: p65 levels in nuclear and cytosolic fractions obtained from cells in NG or HG and treated with or without TNF-α. Equal amounts of protein (10 µg each) were loaded in the lanes. Results shown are representative of three experiments.

els were greater in HG. Furthermore, TNF-α , PDGF, or EGF treatment led to higher nuclear levels of p65 in HG than in NG, as quantitatively depicted in the bar graph below. This was similar to the increased NF-κB DNA binding activity seen under HG conditions induced by these same agents.

We also examined nuclear and cytosolic levels of p50 under these conditions. Figure 8 shows that basal levels of p50, unlike p65, were actually lower in the unstimulated HG nuclear extracts. TNF-α treatment increased nuclear p50 levels in NG as well as in HG to similar extents. No significant changes in p50 levels were noted in the cytosolic fractions obtained after these treatments.

Effect of HG on the transcriptional activity of the human VCAM-1 promoter. TNF-α–induced activation of VCAM-1 gene expression in endothelial cells is mediated, at least in part, transcriptionally through two NF-κB DNA binding elements located at coordinates –77 and –63 (13). To evaluate the functional significance of our observations that HG increased basal and TNF-α–induced DNA binding to an oligonucleotide containing two tandem sequences derived from the human VCAM-1 promoter, we examined whether VCAM-1 transcription was regulated under these conditions. For these studies, the chimeric reporter gene p85VCAMCAT, containing coordinates –85 to +12 of the human VCAM-1 promoter (13), was transiently transfected into PVSMCs that had been cultured for one to two passages in HG. This reporter gene construct contains two NF-κB –like DNA binding elements at –77 and –63 that play an important role in the redox-sensitive cytokine and noncytokine activation of the VCAM-1 promoter (13,28,29,34). Transcriptional activity was assayed by the accumulation of CAT.

Figure 9*A* shows that the addition of TNF-α led to the clear induction of VCAM-1 promoter activity using the p85VCAM-CAT construct in VSMCs cultured in NG as well as in HG. However, the effects of TNF-α were much greater in the cells cultured in HG than in NG. Densitometric quantitation in the bar graph in Fig. 9*B* reveals a significant twofold increase in TNF-α–induced transcriptional activation of the VCAM-1 promoter construct in HG compared with NG. There was no significant increase in basal CAT activity in the unstimulated HG cells relative to NG. These results suggest that the augmented effect of TNF-α on NF-κB activation under HG conditions is associated with increased VCAM-1 promoter activity. As a positive control for the reporter assay, the PVSMCs were also transfected with the constitutively active and highly expressed pSV_2CAT construct containing simian virus 40 promoter and enhancer elements (results show in Fig. 9*A* on the right).

Effect of increasing glucose concentrations on basal and TNF-α–induced superoxide generation. To determine whether HG can lead to increased oxidant stress in the VSMCs, we examined the levels of the reactive oxygen species superoxide in the VSMCs cultured under NG versus 12.5 and 25 mmol/l glucose conditions for 1 week. Superoxide levels were quantitated by the lucigenin chemiluminescence assay. Increasing glucose levels to even 12.5 mmol/l led to a significant increase in superoxide levels ($27.5 \pm 4.5 \times 10^3$ cpm/0.5×10^6 cells vs. $13.2 \pm 1.6 \times 10^3$ cpm/0.5×10^6 cells in NG, $P < 0.01$ vs. NG by paired Student's t test), which were further increased in cells cultured in 25 mmol/l glucose (HG) ($32.4 \pm 2.8 \times 10^3$ cpm, $P < 0.001$ vs. NG control). Treatment of the cells with TNF-α led to a significant increase in superoxide production in the NG cells ($22.2 \pm 2.4 \times 10^3$ cpm, $P < 0.02$ vs. NG control), and these stimulatory effects of TNF-α were further increased at 12.5 mmol/l glucose concentrations ($36.3 \pm 5.8 \times 10^3$ cpm, $P < 0.02$ vs. 12.5 mmol/l basal and $P < 0.04$ vs. 5.5 mmol/l TNF-α by ANOVA). At 25 mmol/l (HG) concentrations, TNF-α could further augment superoxide production to only a small extent over the high basal levels ($34.6 \pm 2.8 \times 10^3$ cpm/0.5×10^6 cells, $P < 0.01$ vs. NG TNF-α). Similar results were also observed in cells cultured for two to four passages in HG, and in these experiments, the effect of TNF-α was significantly greater in 12.5 as well as in 25 mmol/l glucose relative to NG. These results indicate that

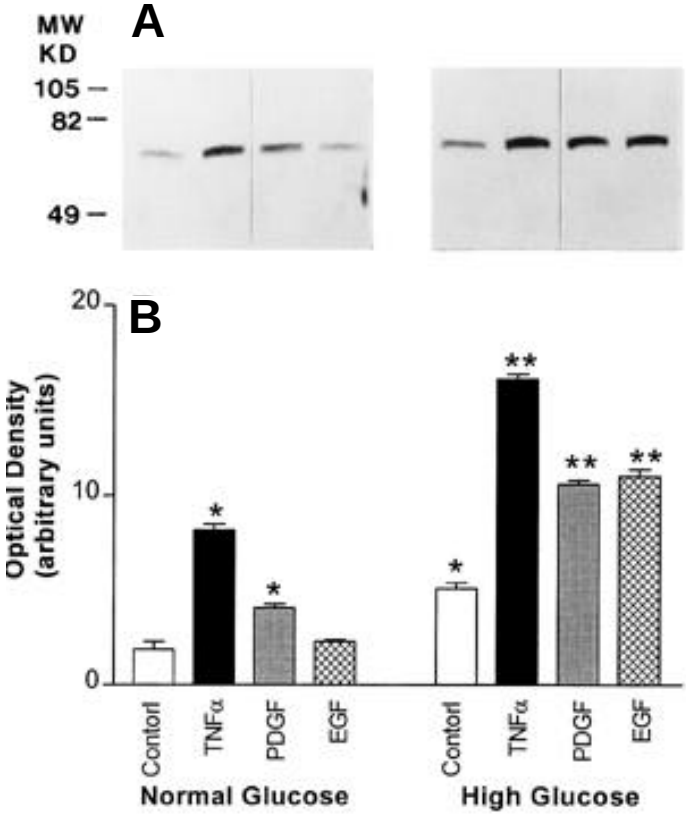

FIG. 7. Regulation of nuclear levels of p65 subunit of NF-κB by growth factors or cytokines. PVSMCs in NG or HG were treated for 3 h with PDGF, EGF (10 ng/ml each), or TNF-α (5 ng/ml). Nuclear proteins (10 μg each) were subjected to immunoblotting to detect p65. *A*: Representative immunoblot. *B*: Bar graph showing the densitometric quantitation of the results (means ± SE) from three experiments. *P < 0.02 vs. NG control, **P < 0.01 vs. HG control (by ANOVA).

elevated glucose concentrations can lead to increased oxidant stress by the production of superoxide.

Effect of a PKC and tyrosine kinase inhibitor on HG-induced NF-κB. We next examined whether PKC or tyrosine kinase activation may be involved in HG-induced NF-κB activation. Cells growing in NG or HG were treated with or without a specific PKC inhibitor, calphostin C (100 nmol/l), or a tyrosine kinase inhibitor, genistein (1 μmol/l). Figure 10 shows that the PKC inhibitor calphostin C could completely block the NF-κB activity induced by HG. In contrast, genistein, a tyrosine kinase inhibitor, did not have any effect. Because HG has been shown to increase PKC activity in VSMCs, these results suggest that PKC activation may be a potential mechanism for the increased NF-κB activity observed under HG conditions.

DISCUSSION

Several lines of evidence indicate that the NF-κB/Rel transcription factors play an important role in atherosclerosis (7–9,33). However, the role of NF-κB activation in hyperglycemia-induced diabetic vascular disease is not clear. We have shown for the first time that VSMCs cultured under HG conditions have significantly higher NF-κB activity in the basal state and in response to cytokines than those cultured under NG conditions. Increased NF-κB activation in the basal state and in response to TNF-α was observed at elevated glucose concentrations ranging from 10 to 25 mmol/l, thus indicating the potential relevance of the observations in leading to diabetic complications. Furthermore, the effects of HG were evident as early as 2 days of treatment and remained sustained for four passages in HG (30–40 days). VSMC growth factors and chemotactic agents, such as PDGF, IGF-I, and EGF could also activate NF-κB, and their effects were enhanced under HG conditions. The observed effects were specific for NF-κB, since the DNA binding pattern of two other transcriptional factors, Sp1 and AP-2, were not similarly regulated.

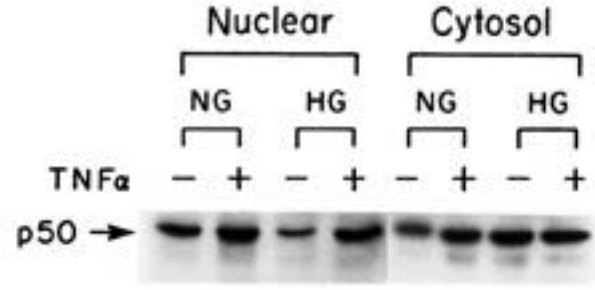

FIG. 8. Expression of the p50 subunit of NF-κB in VSMCs cultured in NG versus HG. VSMCs growing in NG or HG for two passages were serum-depleted for 24 h and then treated with or without TNF-α (5 ng/ml) for 3 h in medium containing 0.2% BSA only. p50 levels in nuclear and cytosolic fractions (20 μg each) were detected by immunoblotting with an antibody specific for p50. Results shown are representative of three experiments.

The augmented TNF-α–induced NF-κB activation in HG was associated with increased TNF-α–mediated VCAM-1 promoter activation in HG relative to NG. In unpublished studies, we have shown that this was associated with significant increases in TNF-α–induced VCAM-1 protein expression in human VSMCs cultured in HG relative to those cultured in NG. These studies were performed in human VSMCs by a specific enzyme-linked immunosorbent assay, since currently, no antibodies to PVCAM-1 are commercially available. These results illustrate the functional significance of our observations. The lack of increased VCAM-1 promoter transactivation in the basal state in unstimulated HG cells, in spite of increased NF-κB activation compared with NG-unstimulated cells, indicates that other NF-κB–activated genes may also be regulated by HG. This is in agreement with earlier observations that HG does not directly increase VCAM-1 expression in endothelial cells (35). HG-induced NF-κB activation may also play a functional role in the accelerated proliferation of VSMCs cultured in HG relative to NG (21,36), since NF-κB activation occurs during VSMC growth (25,26).

We observed that the NF-κB specific protein DNA complexes induced in the VSMCs by HG and TNF consisted mainly of p50/p65 dimers and not the other subunits (cRel, RelB, or p52), similar to other reports on VSMCs (26,37). The p65 subunit has been shown to be the key component of the NF-κB complex (38). Antisense oligonucleotides to p65 blocked human VSMC proliferation and adherence and also inhibited neointima formation in balloon angioplasty–treated rat carotid arteries (33). Further, activated NF-κB was detected in human atherosclerotic lesions in VSMCs, macrophages, and endothelial cells using an antibody that recognizes the nuclear localization signal on p65 (9). In the present studies, we observed that the nuclear levels of p65 were

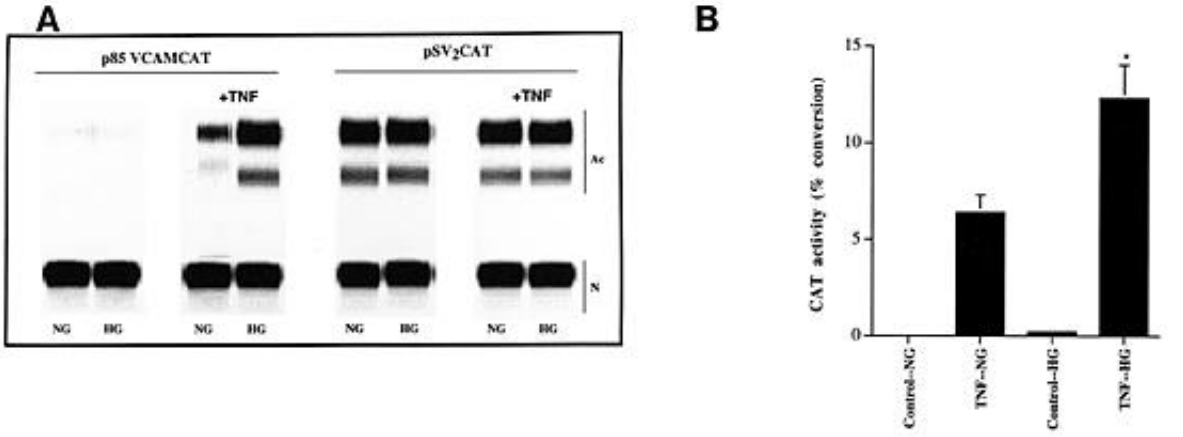

FIG. 9. Transcriptional activation of the human VCAM-1 promoter by TNF-a. PVSMCs cultured for two passages under NG or HG conditions were transfected with either 10 µg of p85VCAMCAT or pSV2CAT as described in methods. After an 18-h exposure to TNF-α (5 ng/ml), cell extracts were prepared and CAT activity determined (Ac, acetylated; N, nonacetylated chloramphenicol). *A*: Representative analysis. *B*: Bar graph showing the quantitation of data from four separate experiments. *P < 0.05 vs. TNF-α in cells cultured in NG.

increased by HG as well as by cytokines and growth factors, suggesting that the augmented NF-κB activation by these agents may arise from the increased nuclear translocation of the p65 subunit.

On the other hand, nuclear p50 levels were actually lower in the basal state in HG nuclear extracts while still being increased by TNF-α treatment under both HG and NG conditions. This is in agreement with observations that p65 is a powerful activator of the VCAM-1 promoter while p50, although it can increase expression, is a poor transactivator, and higher levels of p50 can lead to transcriptional repression (38).

Activation of the reduction-oxidation sensitive transcriptional factor NF-κB can lead to the expression of genes for several adhesion molecules including VCAM-1 (13,28,39), which mediates the recruitment and retention of monocytes in the subendothelial space (a key early step in the atherosclerotic process). VCAM-1 expression in VSMCs was shown to be upregulated by treatment with cytokines (40,41), and certain growth factors could induce NF-κB in the nuclei of cultured rat aortic VSMCs (42). Our present results demonstrating increased activation of NF-κB by growth factors and cytokines under HG conditions indicate a potential mechanism for the accelerated vascular dysfunction observed in diabetes, since the enhanced NF-κB activation could lead to increased transactivation of key genes involved in these pathological disorders.

The biochemical mechanisms by which cytokines and growth factors and HG activate NF-κB have not been fully investigated in the present studies. However, HG as well as growth factors such as angiotensin II and PDGF can induce the production of oxidant signals such as superoxide and hydrogen peroxide (32,43,44). Therefore, the induction of oxidant stress may represent a common mechanism linking NF-κB activation by these factors. In support of this, we observed that culture of the VSMCs under HG conditions led to significantly increased production of superoxide. Superoxide levels after TNF-α treatment were also greater in the cells treated with elevated glucose relative to NG. However, an antioxidant, *N*-acetylcysteine, could not significantly attenuate either HG- or TNF-α–induced NF-κB in VSMCs (our unpublished observations) unlike that observed

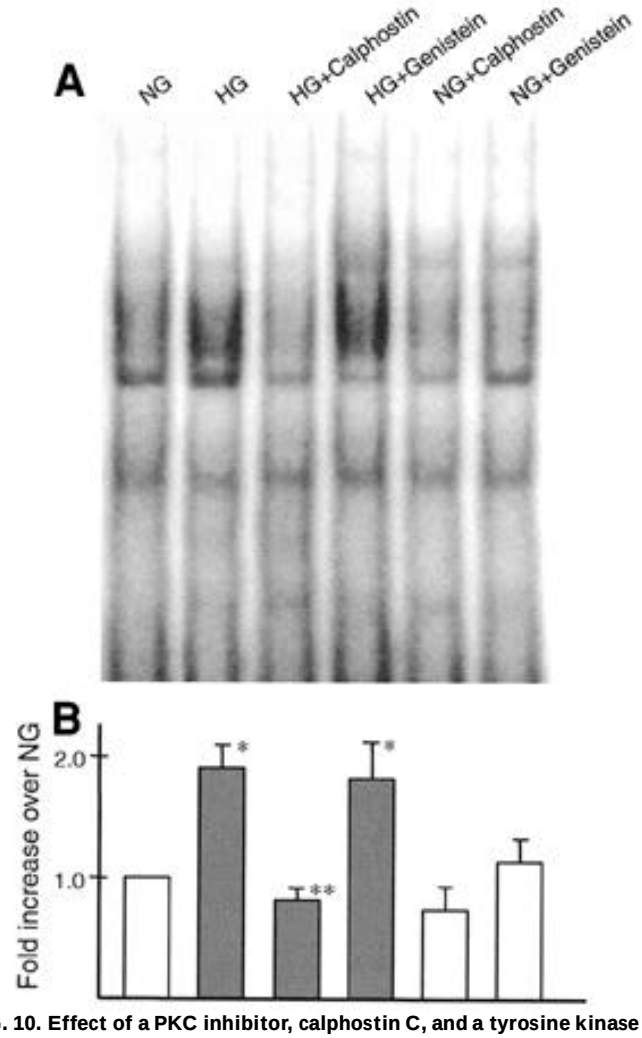

FIG. 10. Effect of a PKC inhibitor, calphostin C, and a tyrosine kinase inhibitor, genistein, on NF-κB activation in VSMCs. Serum-starved cells were cultured in NG or HG in the presence or absence of the PKC inhibitor calphostin C (100 nmol/l) or the tyrosine kinase C inhibitor genistein (1 µmol/l). EMSAs were then run on the nuclear extracts from these cells. *A*: Representative EMSA. *B*: Bar graph results are means ± SE from three experiments. *P < 0.01 vs. NG, **P < 0.001 vs. HG (by ANOVA).

in endothelial cells (28). However, additional studies with other antioxidants are necessary, since our unpublished observations show that hydrogen peroxide can directly increase NF-κB activity in VSMCs. It should be noted that in VSMCs, antioxidants have been reported to have either inhibitory effects or no effects on NF-κB activation (25,45,46). A recent study showed that superoxide dismutase inhibited PDGF- but not IL-1β–induced NF-κB and monocyte chemotactic protein (MCP)-1 expression in VSMCs (45). Hence, the presence of oxidant stress alone may not always be sufficient for HG- and cytokine-induced NF-κB in VSMCs. Alternatively, other NF-κB–activated genes besides VCAM-1, such as MCP-1, may be responsive to oxidant stress in this context.

Several lines of evidence have implicated increased PKC activity as a key player in the pathological effects of HG (16). In the present studies, we observed that a PKC inhibitor could significantly block HG-induced NF-κB, suggesting that increased PKC activity by HG may be a key mechanism for the increased NF-κB under HG conditions. In contrast, a tyrosine kinase inhibitor could not attenuate HG-induced NF-κB activation, suggesting that tyrosine kinase activation may not play a role in HG effects on NF-κB.

Prolonged hyperglycemia can also lead to the formation of AGEs (17,18). Increased AGE formation and action under HG conditions may also be responsible for our present observations. Indeed, very recently, AGEs were shown to directly activate NF-κB in VSMCs (47), and we have also confirmed this in unpublished studies. Our observations that NF-κB activation occurs during short-term as well as chronic HG treatments of VSMCs suggests that the early effects may be mediated by early glycation products while the sustained effects may arise from the action of AGEs.

In summary, these results suggest that increased basal as well as growth factor– and cytokine-induced NF-κB activation in VSMCs under hyperglycemic conditions may represent a key mechanism for the production of VSMC factors mediating the accelerated vascular disease observed in diabetes.

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