Molecular Mechanisms of Tumor Necrosis Factor α Gene Expression in Monocytic Cells via Hyperglycemia-induced Oxidant Stress-dependent and -independent Pathways

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Increased oxidative stress has been reported in vivo in the diabetic state via the production of reactive oxygen species (ROS). Such stress is bound to play a key role on activation of circulating monocytes, leading to the accelerated atherosclerosis observed in diabetes. However, the exact molecular mechanisms of monocyte activation by high glucose is currently unclear. Here, we demonstrate that chronic high glucose (CHG) causes a dramatic increase in the release of the inflammatory cytokine tumor necrosis factor α (TNFα), at least in part through enhanced TNFα mRNA transcription, mediated by ROS via activation of transcription factors nuclear factor κB (NF-κB) and activating protein-1 (AP-1). TNFα accumulation in the conditioned media was increased 10-fold and mRNA levels were increased 11.5-fold by CHG. The following observations supported that both NF-κB and AP-1 mediated enhanced TNFα transcription by CHG: 1) A 295-base pair fragment of the proximal TNFα promoter containing NF-κB and AP-1 sites reproduced the effects of CHG on TNFα transcription in a luciferase reporter assay, 2) mutational analyses of both NF-κB and the AP-1 sites abrogated 90% of the luciferase activity, 3) gel-shift analysis using the binding sites showed activation of NF-κB and AP-1 in CHG nuclear extracts, and 4) Western blot analyses demonstrated elevated nuclear levels of p65 and p50 and decreased cytosolic levels of IκBα in CHG-treated monocytes. That ROS acted as a key intermediate in the CHG pathway was supported by the following evidence: 1) increased superoxide levels similar to those observed with PMA or TNFα, 2) increased phosphorylation of stress-responsive mitogen-activated protein kinases p38 and JNK-1, 3) counteraction of the effects of CHG on TNFα production, the 295TNFFluc reporter activity, activation of NF-κB, and repression of IκBα by antioxidants and p38 mitogen-activated protein kinase inhibitors. The study suggests that ROS function as key components in the regulatory pathway progressing from elevated glucose to monocyte activation.

Cellular redox state has been shown to play an important role in the pathogenesis of cardiovascular disease including atherosclerosis, the rate of which is higher in diabetics (1–3). Hyperglycemia in the blood stream could generate free radicals and peroxide species by slow “autoxidation” of glucose, causing oxidative stress to circulating monocytes (4, 5). Furthermore, glycosylation of low density lipoprotein increases its susceptibility to oxidation, generating byproducts in circulation that preferentially accumulate in foam cell-generating monocytes/macrophages (6, 7). Additionally, soluble advanced glycation end products (AGEs) present in the blood stream could also generate reactive oxygen species (ROS) (8–10). AGEs deposited in the arterial walls generate free radicals capable of oxidizing vascular lipids and accelerating atherogenesis in hyperglycemia (9, 11).

As peripheral blood glucose levels increase in hyperglycemia, there is simultaneous rise in intracellular glucose levels, utilizing the sorbitol pathway and altering the redox balance inside the cells. Hyperglycemia also leads to increased NADH/NAD⁺ ratio, thereby decreasing the availability of NAD⁺ as a co-factor for other metabolic events (12–14). The redox changes induced by hyperglycemia, AGEs, and lipid peroxidation have been shown to alter cellular functions via activation of key signal transduction pathways involving MAPKs such as ERK 1/2, JNKs, and p38 (15–18). High glucose and diabetes have been shown to specifically activate p38 MAPK via ROS intermediates in smooth muscle cells (19–20), and oxidant stress has been shown to incite macrophage spreading via the p38 MAPK pathway (21). In addition, production of inflammatory cytokines such as TNFα and interleukin-6 by activated rat smooth muscle cells was regulated by the p38 MAPK pathway (22). Activation of the p38 MAPK has been observed in a number of physiological responses such as apoptosis of myocardial cells (25) and adipogenesis in 3T3-L1 cells (23). Altered NADH/NAD⁺ ratio caused by hyperglycemia results in de novo synthesis of diacylglycerol and activation of various protein kinase C (PKC) isoforms in cell/tissue-type and stimuli-specific manner (7, 25, 44). That hyperglycemia induced ROS may function as a key intermediate leading to the activation of PKC has been shown in many cell types of human and porcine origin.

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The abbreviations used are: AGE, advanced glyated end product; ROS, reactive oxygen species; O₂⁻, superoxide; TNFα, tumor necrosis factor α; hTNFα, human TNFα; HG, high glucose; CHG, chronic HG; NG, normal glucose; NF-κB, nuclear factor κB; IκBα, inhibitor κB; AP-1, activating protein-1; PMA, phorbol 12-myristate 13-acetate; MAPK, mitogen-activated protein kinase; JNK-1, c-Jun amino-terminal kinase; PKC, protein kinase C; EMSA, electromobility shift assay; ERK 1/2, extracellular signal-regulated kinase; LPS, lipopolysaccharide; NAC, N-acetyl-L-cysteine; PDTC, pyrrolidine dithiocarbamate; RT-PCR, reverse transcriptase-polymerase chain reaction; ICS, internal control standard; LCA, lucigenin chemiluminescence assay.
The inflammatory cytokine human tumor necrosis factor alpha (hTNFα) is produced by activated monocytes in response to a variety of signals including stress response, phorbol esters, cytokines, endotoxin, and substrate adherence (27–31). TNFα gene expression is regulated both at the levels of transcription and post-transcription. Elevated levels of TNFα and other inflammatory cytokines have been detected in atherosclerotic plaques of diabetic and nondiabetic patients (32).

The role of monocytes in increased foam cell formation in diabetic patients is well established (25). Hyperglycemia-induced oxidative stress, further accentuated by the inactivation of superoxide dismutase (12), along with soluble AGEs and products of lipid peroxidation possibly serve as key activators of circulating monocytes via the activation of upstream kinases, leading to induction of inflammatory gene expression. However, the signaling kinases or transcription factors specifically involved in high glucose-induced monocyte activation leading to the production of the inflammatory cytokine TNFα are still unclear and are the focus of the present study.

Activation of genes in response to inflammatory stimuli has been shown to involve coordinated participation of transcription factors NF-κB and AP-1. Regulation of many inflammatory cytokines, tissue factor, and matrix metalloproteinases involve dual transcriptional regulation by NF-κB and AP-1 (33–36). NF-κB/Rel proteins are heterodimeric transcription factors retained in the cytoplasm of unstimulated cells by the inhibitory subunit IkB, the NF-κB/IκB forming an inactive ternary complex (37). Stimulation with stress-inducing agents or other proinflammatory mediators causes rapid phosphorylation, ubiquitination, and degradation of IkB-subunit, allowing translocation of NF-κB to the nucleus (37). NF-κB then induces transcription of several genes, including that of its inhibitor IkB (38). Another transcription factor regulated by cellular stress is AP-1, a transacting molecule consisting mainly of homodimers of Jun or heterodimers of Fos and Jun (39). The hTNFs gene has in its promoter region canonical binding sites for transcription factors NF-κB and AP-1 (40).

Since the exact mechanism for the activation of monocytes leading to inflammatory cytokine production by hyperglycemia is currently unclear, we evaluated some of the key molecular and cellular events leading to TNFα secretion by high glucose. Our study suggests that CHG-induced monocyte activation, as evidenced by increased TNFα expression, was regulated at least in part through increased TNFα mRNA transcription. The process involved ROS-dependent and -independent pathways, requiring coordinate activation of both p38 MAPK and PKC as upstream kinases and NF-κB and AP-1 as downstream transcription factors.

**Materials and Methods**

**Cell Culture—**U937 (monoblastoid) and THP-1 (histiocytic) cells were obtained from ATCC and maintained in RPMI 1640 medium containing 7% heat-inactivated fetal calf serum, β-mercaptoethanol (50 μM), HEPES (10 mM), glutamine (2 mM), streptomycin (50 μg/ml), penicillin (50 units/ml), and 5.5 mM glucose (NG). For chronic high glucose (CHG) conditions, cells were cultured in 12.5, 15, or 25 mM glucose for 2 days before being depleted of serum and treated with various agents as indicated under “Results.” High glucose (HG) culture was done in 15 mM glucose for 18 h. The HG condition was used to culture peripheral human monocytes for TNFα proctest and for 295TNFLuc luciferase activity studies. CHG- or HG-treated cells were washed and resuspended in depletion medium containing 0.5% bovine serum albumin for 18 h or for other periods as indicated prior to stimulation.

**Preparation of Human Monocytes—**Fresh human monocytes were obtained from healthy donors using an approved Institutional Review Board protocol and isolated as described previously (41). Autologous serum was used for the attachment and culture of the monocytes.

**Lucigenin Chemiluminescence Assay (LCA)—**This was performed as described earlier to measure superoxide anion (O2⁻). Briefly, U937 or THP-1 cells were cultured in NG or CHG, depleted of serum using depletion media, and prepared for the assay. Cells were treated with TNFα (5 ng/ml) or PMA (10 ng/ml; positive control) for 60 min, washed in a balanced salt solution, and resuspended at 2 × 10⁶ cells/ml in aerated balanced salt solution. 1 × 10⁶ cells/ml was used for the assay. O₂⁻ was measured in intact cells by LCA. Cells from various treatments were added to a scintillation vial containing lucigenin (500 μM) in the aerated balanced salt solution. Photon emission was measured for 10 min using the Beckman LS6500 Multipurpose Scintillation counter measuring single photon emission. First, photon emission was measured using a buffer blank and dark-adapted lucigenin, and the blank reading was subtracted from the sample reading. A standard curve was generated using xanthine and xanthine oxidase. Superoxide dismutase (100 units/ml) was used as an inhibitor for superoxide production. H₂O₂ was added to the buffer blanks or to the NG control cells to determine if the photon emission in the lucigenin assay was induced by O₂⁻ or peroxide species.

**Inhibitors and Reagents—**N-Acetyl-l-cysteine (NAC; 100 μM) was purchased from Calbiochem. It was dissolved in phosphate-buffered saline, and the pH was adjusted to 7.2. Pyrroline dithiocarbamate (PDTC; Sigma; 50–100 μM) was dissolved in water. Me₂SO was used to resuspend the rest of the inhibitors and was added to the control plates. p38 MAPK inhibitor SB202190 (SB, 10 μM) was purchased from Upstate Biotechnology. Mannitol, 3-O-methyl-glycidol, and 2-deoxyglucose were purchased from Sigma.

**Detection of Secreted TNFα in the Culture Supernatant by Specific hTNFα Enzyme-linked Immunosorbent Assay—**U937 or THP-1 cells were cultured in NG or CHG, depleted of serum in depletion media, and cultured in NG, CHG, or NG + PMA for another 24 h. Quantitative detection of hTNFα in conditioned media was performed using a specific antibody sandwich Cytoscreen™ enzyme-linked immunosorbent assay (ELISA) from BIOSOURCE International using the manufacturer’s suggested instructions. Known concentrations of hTNFα were used to generate the standard curves. Supernatant from NG-cultured cells treated with PMA (10 ng/ml) for 24 h served as the positive control. Streptavidin-horseradish peroxidase served as the detection system. For each experiment, duplicate samples were measured. Data were represented as the mean (pg/10⁶ cells) ± S.E. The assay was linear between 15.6 pg/ml and 1000 pg/ml.

**Quantification of TNFα Message by Competitive RT-PCR Assay—**TNFα message was measured in U937 cells using the Quantitative RT-PCR Cytoexpress detection kit from BIOSOURCE International. Cells were cultured and depleted as in the preceding paragraph above, and RNA was extracted from CHG or NG cells stimulated for 4 h with various concentrations of TNFα (0.25, 0.5, 1, or 5 ng/ml) or PMA (10 or 50 ng/ml). RNA was reverse-transcribed to cDNA using murine leukemia virus reverse transcriptase. A known copy number of exogenously synthesized DNA, known as the internal control standard (ICS) was mixed with sample cDNA before PCR amplification. The ICS contained PCR primer binding sites similar to the TNFα cDNA and a unique capture-binding site to distinguish the ICS amplicon from the TNFα amplicon. In samples containing ICS, two amplicon bands were visible following PCR amplification. The 382-base pair TNFα band and the 432-base pair ICS band. After amplification, the amplicons were hybridized to the ICS or TNFα-specific oligonucleotide-coated wells. Biotinylation of an original primer allowed streptavidin coupled to biotin to be used as the detection system. The signal generated in the hybridization reaction was proportional to the number of amplicons present in the starting cDNA. Since the ICS (known copy number) was amplified at the same frequency as the TNFα cDNA, it served to determine the copy number of TNFα cDNA in each sample (see Equation 1).

Copy number of TNFα message = 2 × known ICS copy number

\[ A \text{ in TNFα wells} \times A \text{ in ICS wells} \]

(Eq. 1)

**Plasmids and Luciferase Reporter Gene Assays—**The 295TNF Luciferase (295TNFLuc) deletion construct from the human TNFα promoter was a kind gift from Dr. James S. Economou, UCLA (44). 5 × 10⁵ U937 or THP-1 cells were depleted of serum 2 h before transfection. The cells were transfected with 2 μg of the 295TNFLuc deletion construct and co-transfected with 0.2 μg of reporter plasmid (RSVβGal) using the Transfection kit and the procedures as described by the kit manufacturer.

Reference:
(22, 26). PKC-dependent and -independent activation of p38 MAPK pathway was observed in smooth muscle cells (19) and mesangial cells, respectively (24).
Effectively cultured in NG or CHG for 24 h, washed, depleted for 4 h, and transfected with 293TNFluc plasmid overnight. On day 3, cells were lysed and proteins were extracted with NP-40 or formaldehyde. Western blots were quantified with the Alpha Imager documentation system using the Alpha Imager 3.24 software.

**Experimental Methods**

The Western blot analysis was performed using the Quick-change™ site-directed mutagenesis kit from Stratagene using the manufacturer’s suggested directions. The NF-κB (GAGGCTCCCTG) and AP-1 (TGAATGA) sites were used for the EMSA. The sequence for the NF-κB site was: GAGGCTCCCTG. The sequence for the AP-1 site was: TGAATGA. The EMSA was performed according to published protocols (47).

**Preparation of Nuclear and Cytosolic Extract for Gel-shift (Electromobility Shift Assay) and Western Analyses**

Nuclear and cytosolic extracts were made using lysis buffer containing 1% Triton, 0.5% sodium deoxycholate, 100 mM HEPES, 10 mM NaCl, 10 mM EGTA, and 25 mM NaF. For these experiments, cells lysates were made using lysis buffer described earlier (20). All Western blots were developed using the ECL detection system (Amersham Pharmacia Biotech), following the protocol suggested by the manufacturer. Anti-rabbit horseradish peroxidase-conjugated secondary antibody was used as the loading control. Phosphorylated ERK 1/2, JNK-1, and p38 were detected by Western blot analysis using a Turner TD-20e luminometer measuring luciferase activity. The luciferase assay was performed using the firefly luciferase kit (Promega) using a Turner TD-20e luminometer measuring light intensity over a 5-log range. Results from the luciferase assay were normalized to β-galactosidase levels, and relative luciferase units were determined. The results were reported as fold stimulation over XP-1 control plasmids. Concentrations of inhibitors used for the study are listed below “Inhibitors and Reagents” of this section.

**RESULTS**

**CHG Induces Oxidant Stress in Monocytes**

CHG increased reactive oxygen species in monocytic cells detected by LCA. To investigate whether CHG increased ROS in monocytic cell lines (U937 and THP-1), we examined the effect of CHG culturing alone on superoxide (O2−) production. CHG increased O2− levels significantly (p < 0.005) over NG-cultured U937 cells (2.3 ± 0.87-fold; Fig. 1A). O2− levels generated by CHG were comparable with that observed in NG cells following treatment with the inflammatory cytokine TNFα or phorbol ester PMA (positive control) for 1 h. Treatment of cells with superoxide dismutase before measuring O2− levels by LCA quenched any detectable O2− produced in CHG-, TNFα-, or PMA-treated cells. Adding H2O2 to NG cells did not increase chemiluminescence counts, indicating that O2− but not peroxide was the contributor for the photon emission. THP-1 cells generated similar trends but had higher levels of O2− than U937 cells.

**Elevated Phosphorylation of Stress-responsive MAPks by CHG, Confirming Oxidant Stress**

An important measure of oxidative stress is the activation of upstream stress-responsive MAPks. We therefore examined the effects of CHG on phosphorylation of p38 (pp38), JNK-1 (pJNK-1), and ERK 1/2 (phosphorylated) using Western blot analysis. Fig. 1B, top panel 1, shows a representative immunoblot probed with antibodies to pp38. In the middle panel, the same blot was stripped and re-probed with pJNK-1 antibody, and in the bottom panel, the blot was stripped further and probed with nonphosphorylated p38 antibody serving as a loading control. THP-1 cells cultured in CHG showed a striking increase in basal levels of pp38 (Fig. 1C, 1.6-fold) and pJNK-1 (Fig. 1D, 2.3-fold) over NG controls. Treatment of cells with TNFα for 5 or 10 min (Fig. 1C) showed a stronger increase in pp38 levels in NG (2.1-fold) compared with CHG (0.6-fold). On the contrary, the pJNK-1 levels (Fig. 1D) following TNFα treatment showed a much stronger and faster increase (2.5-fold) in CHG compared with NG (1.7-fold). The activation profile of the two stress-responsive kinases in CHG showed positive correlation with O2− data. TNFα-induced elevated pp38 and pJNK-1 levels in CHG were additive. In contrast, the third member of the MAPk family, ERK 1/2, which generally is responsive to stress and mitogenic stimuli, showed no activation by either high glucose or TNFα in these monocytic cells. CHG or TNFα showed similar trends in U937 cells as THP-1. Increased superoxide levels and elevated phosphorylation of stress-responsive MAPks, p38, and JNK-1 by CHG demonstrate that ROS induced by high glucose could potentially contribute to downstream signaling via activation of MAPks.

**Elevated Levels of TNFα Accumulate in Conditioned Media of U937, THP-1, or Normal Human Monocytes Cultured in CHG**

TNFα is a potent cytokine involved in inflammation, and elevated levels of TNFα are seen in atherosclerotic plaques of diabetics. We therefore evaluated if culturing monocytic cells in CHG could lead to increased TNFα accumulation in conditioned medium. CHG alone induced a dramatic increase in TNFα accumulation in conditioned media of U937 (Fig. 2A, 10.7-fold), THP-1 (Fig. 2B, 8.17-fold), and normal isolated human monocytes (Fig. 2C, 10.2-fold) compared with their NG counterparts. PMA was used as a positive control. To determine whether the effects of CHG (15 mM)-induced TNFα accumulation was due to increased osmolality of CHG, mannitol (9.5 mM) in NG was used as a control. To evaluate if glucose metabolism was required for the increased TNFα secretion by CHG, 3-O-methyl glucose (9.5 mM) in NG was tested.
cells in mannitol, or 3-O-methyl glucose showed comparable levels of TNFα in the conditioned media as NG controls. These results confirmed that the effect of CHG on elevated TNFα accumulation was not due to hyperosmolality of CHG and that glucose metabolism was essential for the elevated levels of TNFα.

TNFα Is Transcriptionally Regulated in U937 Cells Cultured in CHG

To evaluate if CHG-induced TNFα is regulated transcriptionally in monocytic cells, we used competitive RT-PCR to monitor the levels of TNFα message. Since TNFα is regulated both by autocrine and paracrine pathways, we compared the levels of TNFα message induced by the cytokine to that induced by CHG. Ethidium bromide staining of products from RT-PCR analysis (Fig. 3A) demonstrated that the low levels of TNFα (0.25, 0.5, or 1.0 ng/ml), similar to that secreted in conditioned media by CHG culturing, could stimulate TNFα message in an autocrine fashion (Fig. 3A, top panel, lanes 5–7). The peak of this stimulation was at 1 ng/ml. The addition of higher levels of TNFα (5 ng/ml) did not further increase the levels of the cytokine message (Fig. 3A, top panel, lane 8). Interestingly, CHG induced TNFα message at levels similar to that induced by 0.25–0.5 ng/ml TNFα (Fig. 3A, top panel, lanes 11 and 12). PMA at 10 ng/ml induced about 2.4-fold higher message compared with CHG (Fig. 3A, top panel, lane 4) and served as the positive control for TNFα message induction. Glyceraldehyde-3-phosphate dehydrogenase was used to check the integrity of RNA (Fig. 3A, bottom panel). TNFα cDNA was also amplified using competitive PCR in tubes containing known copy numbers of ICS and hybridized to wells containing either the ICS oligonucleotide or the TNFα-specific oligonucleotide to determine copy number of TNFα message in the different cDNA samples (Fig. 3B). At low levels (0.25–1 ng/ml), TNFα showed a dose-dependent increase in the copy number of specific TNFα message. Copy number of TNFα message induced by CHG was comparable with that induced by PMA (0.5 ng/ml) (Fig. 3B). The competitive RT-PCR data in U937 cells suggest that secreted TNFα, at levels induced by CHG, could act through an autocrine loop to transcriptionally regulate further TNFα message in monocytic cell lines. THP-1 cells showed a similar trend as U937 cells but gave a higher copy number of TNFα message following different stimulations (data not shown). Data from RT-PCR studies indicate that the regulation of TNFα by CHG in U937 and THP-1 cells is controlled, at least in part, transcriptionally, although post-transcriptional control may also play a significant role in TNFα regulation.
Identification of Cis-elements Involved in CHG Mediated Increased Transcriptional Regulation of TNFα

TNFα is regulated both transcriptionally and post-transcriptionally in response to various stimuli. In our study, the competitive RT-PCR data demonstrated that TNFα expression in monocytic cells has a transcriptional component in response to CHG, and therefore, some of the cis-elements in the TNFα promoter involved in the induction of this inflammatory cytokine by CHG was evaluated. The 295TNFluc promoter construct selected for our study has been used to study the cis-elements involved in TNFα promoter regulation following induction by various stimuli such as TNFα, PMA, LPS, and cyclosporin A (29, 30, 39). U937 cells cultured in NG or CHG were transfected with the 295TNFluc plasmid and cotransfected with the β-galactosidase as internal control, and normalized luciferase activity was measured following stimulation with or without TNFα for 18 h. CHG stimulated similar luciferase activity as TNFα-treated NG cells (Table I). However, the effect of CHG plus TNFα on luciferase activity was additive over CHG alone (Table I), possibly suggesting the involvement of more than one pathway in transcriptional regulation of TNFα by CHG and TNFα. Specificity for CHG-stimulated luciferase activity was determined by using mannitol (control for osmolality), 3-O-methyl glucose and 2-deoxyglucose (control for glucose metabolism). All of the controls demonstrated near NG levels of luciferase activity (Table I). In some experiments luciferase activity was measured in transfected NG cells stim-
ulated with HG (15 mM) for 18 h. HG in the stimulation phase showed a similar increase in luciferase activity as CHG. These results demonstrate that 295 base pairs of proximal promoter region, adjacent to the transcriptional start site, in the TNFα promoter could reproduce the effects observed on the TNFα message by competitive RT-PCR following stimulation by CHG or TNFα in the luciferase reporter assay.

**Mutational Analysis of the NF-κB and AP-1 Sites in the Proximal TNFα Promoter**

The 295TNFα luc plasmid has, in its proximal promoter, consensus binding sites for NF-κB and AP-1 transcription factors (Fig. 3C). Site-directed mutagenesis was used to introduce two point mutations each, at the NF-κB (GGGTTTCC mutated to TGTTTCTCC) and AP-1 sites (TGAATGA mutated to GTAAATGA). Mutation of these sites completely abrogated binding of NF-κB or AP-1 to their respective sites using CHG- or TNFα-stimulated nuclear extracts in EMSA (data not shown). Mutation at the NF-κB site and the AP-1 sites reduced CHG-stimulated 295TNFα luc activity by 71.8 ± 3.73 and 31.5 ± 4.7%, respectively (Table I). Interestingly, double mutations of both the sites abrogated CHG-stimulated luciferase activity by 89.3 ± 4.31%, confirming the critical contributions of NF-κB (major) and AP-1 (minor) in CHG-mediated induction of the TNFα promoter.

**EMSA to Study the Effect of CHG Culturing on NF-κB Activation**

The pleiotropic transcription factor NF-κB has been shown to be responsive to oxidant stress in endothelial cells, smooth muscle cells, and mesangial cells, all of which play a critical role in atherosclerosis (1, 4, 11). Mutational analyses demonstrated NF-κB as a major transcription factor in CHG-stimulated luciferase activity. Therefore CHG- and/or TNFα-mediated activation of NF-κB in monocytic cell lines was confirmed using EMSA, performed with a 22-base pair oligonucleotide containing the NF-κB site from the hTNFα promoter. Nuclear extracts from U937, THP-1, and fresh human monocytes (from healthy donors) were used for EMSA (Fig. 4, A–D). Culturing U937 cells in 5.5 mM NG (15 mM CHG), or 25 mM (VHG) glucose alone for 3 days showed a dose-dependent increase in NF-κB binding (Fig. 4A, first, third, and fifth lanes). PhosphorImager quantitation of data from control experiments showed that NF-κB binding was significantly increased (p < 0.05) in 15 mM (2.1 ± 0.83-fold) and 25 mM glucose (3.2 ± 0.68-fold) over NG (Fig. 4C). Representative gels showing the effect of CHG on NF-κB binding are shown in Fig. 4, A and B. Culturing primary human monocytes overnight in 15 mM glucose (HG) also caused a marked increase in NF-κB binding over NG controls (Fig. 4D, first and second lanes). Since CHG leads to accumulation of TNFα in the conditioned media, we evaluated the NF-κB binding response to TNFα in CHG versus NG cells. After 1 h of continuous treatment with TNFα, NF-κB binding in NG and CHG (15 mM) cells were not significantly different (representative gels shown in Fig. 4, A and B). Culturing cells in NG for 3 days also demonstrated p50 and p65 as the major subunits of the NF-κB complex, showing p50 alone, suggesting an autocrine role of this cytokine in CHG-stimulated TNFα gene regulation. The stimulatory effect of CHG and TNFα were specific to NF-κB, because the ubiquitous transcription factor Oct-1 was not affected (panels 2 of Figs. 4, A and B).

**Specificity for CHG-stimulated NF-κB binding was determined by culturing U937 cells in NG plus 9.5 mM mannitol, as a control for osmolality**, with a marked increase in NF-κB binding over NG (Fig. 5, A and B). PhosphorImager quantitation of data from control experiments showed that the basal NF-κB binding in NG (Fig. 5A, lane 4) and CHG (Fig. 5B, lane 4) was significantly increased (p < 0.05) over NG alone, suggesting an autocrine role of this cytokine in CHG-stimulated TNFα gene regulation. The stimulatory effect of CHG and TNFα were specific to NF-κB, because the ubiquitous transcription factor Oct-1 was not affected (panels 2 of Figs. 4, A and B).

**Elements of NF-κB Complex Affected by CHG Culturing and/or TNFα Treatment**

To identify the elements of NF-κB complex affected by CHG or TNFα stimulation, a time course study was performed in U937 cells cultured in NG or CHG following TNFα treatment. **TABLE I**

| Stimulation | Relative luciferase activity | Fold induction over NG control |
|-------------|-----------------------------|--------------------------------|
| NG          | 3.2 ± 1.23                  | 1.0                            |
| NG + 9.5 mM mannitol | 3.6 ± 0.85                  | 1.12 ± 0.6                    |
| NG + 9.5 mM 3-O MG | 2.3 ± 1.14                  | 0.72 ± 0.87                   |
| NG + 9.5 mM 2 deoxy G | 2.6 ± 0.89                  | 0.87 ± 0.89                   |
| HG          | 7.14 ± 1.01                 | 2.92 ± 1.23                   |
| CHG         | 10.14 ± 1.65                | 3.17 ± 1.67                   |
| NG + TNFα   | 11.39 ± 2.15                | 3.56 ± 2.04                   |
| CHG + TNFα  | 16.73 ± 1.78                | 5.23 ± 1.73                   |

* Fold induction over control XP-1 plasmid (data normalized to β-galactosidase).

**TABLE II**

| Stimulation | Percent reduction in luciferase activity |
|-------------|-----------------------------------------|
| NG          | 1.3 ± 2.18                              |
| CHG         | 71.8 ± 3.73*                            |
| NG + TNFα   | 67.8 ± 5.97*                            |
| CHG + TNFα  | 74.3 ± 4.11*                            |

* Data represent the average ± S.E. of five different experiments.

**TABLE III**

| Percent reduction in luciferase activity |
|-----------------------------------------|
| NG          | 1.3 ± 2.18                              |
| CHG         | 71.8 ± 3.73*                            |
| NG + TNFα   | 67.8 ± 5.97*                            |
| CHG + TNFα  | 74.3 ± 4.11*                            |

* Data represent the average ± S.E. of three different experiments.

* Fold induction over control XP-1 plasmid (data normalized to β-galactosidase).
The regulation of the NF-κB complex over time was monitored using EMSA and Western blot analysis. Western blots of nuclear extracts were sequentially probed with anti-p65, anti-p50, anti-IκBα, and anti-Histone H1 (loading control) antibodies, and those of cytosolic extracts were sequentially probed with anti-IκBα, anti-IκBε, and anti-actin (loading control) antibodies with stripping of the blots following each detection. Representative gels from the time course study are shown in Fig. 5, A, C, and E (NG) and B, D, and F (CHG), and some of the key data from the same study are graphically summarized in Fig. 5, G, I, and K (NG) and H, J, and L (CHG).

The time course of NF-κB activation in NG showed a gradual increase in NF-κB binding, with peak binding at 60 min after TNFα treatment. The increased binding persisted for 2 h post-TNFα stimulation (Fig. 5A, panel 1, fifth and sixth lanes) and fell to basal NG levels by 4 h (data not shown). In CHG, NF-κB activation following TNFα treatment was strongly detectable as early as 5 min (Fig. 5B, panel 1, second lane). There was a gradual increase in NF-κB binding, which peaked around 15–30 min following TNFα treatment (Fig. 5B, panel 1, third and fourth lanes) and remained sustained even at 18 h (data not shown). Oct-1 (Fig. 5, A and B, panels 2) served as a control for nuclear extract preparation and was not affected by CHG or TNFα at any time points. Under CHG conditions, basal levels of NF-κB binding were about 2-fold higher compared with basal NG levels (Fig. 5, A versus B, panel 1, first lane). The NF-κB binding data suggest that CHG-cultured U937 cells are primed for a faster responsiveness to TNFα.

To explore the mechanism for this observed increase in NF-κB binding in CHG, we examined the levels of the p50 and p65 subunits in nuclear extracts, since they were the only subunits identified by supershifting. The Western blots from nuclear extracts demonstrated higher p65 levels in CHG compared with the barely detectable levels in NG (Fig. 5, C and D, panel 1, first lane). Basal p50 levels in CHG were also higher (3.25 ± 0.69-fold) compared with NG levels (Fig. 5, C and D, panel 2, first lane). Mimicking the EMSA (Fig. 5, A versus B), the graphical results of the Western analysis (Fig. 5, I versus J) showed significantly higher levels of p65 in CHG compared with NG (p < 0.05) at the early time points (5–15 min) of TNFα treatment. However, at the later time points (60–120 min) of continuous TNFα treatment, p65 levels...
Regulation of TNFα by Hyperglycemia-induced Oxidant Stress

**FIG. 5.** Time course of NF-κB activation in U937 cells following treatment with TNFα. Extracts from the same NG cells were used in A, C, and E, and extracts from the same CHG cells were used in B, D, and F. A, B, and U937 cells were cultured in NG (A) versus CHG (B) as indicated prior to treatment with TNFα for the indicated time points. Glucose concentration was constant throughout the experiment. Cells were harvested at each time point, nuclear extracts were made, and EMSA was performed using probes for NF-κB or Oct-1 as indicated. A and B show basal and TNFα-stimulated DNA binding with NG and CHG-cultured cells, respectively. C and D, analysis of nuclear levels of transcription factors by Western blotting following NG (C) or CHG (D). 10 μg of nuclear extracts from A and B were analyzed by immunoblots using anti-p65 (Panel 1), -p50 (Panel 2), -IκBα (Panel 3), or -histone H1 (Panel 4, loading control) antibodies. The same blots were repeatedly stripped and reprobed. E and F, analysis of cytosolic levels of transcription factors following NG (E) or CHG (F). 10 μg of cytosolic extracts from A and B were analyzed by immunoblotting using anti-IκBα (Panel 1), -IκBβ (Panel 2), -IκBe (Panel 3), or -actin (loading control, Panel 4) antibodies. The same blots were repeatedly stripped and reprobed. G-L, all values are means ± S.E. of three different experiments from A–F, G and H, graphical representation of PhosphorImager counts from A and B (Panel 1). I and J, graphical representation of densitometric quantitation from C and D (Panel 1). K and L, graphical representation of densitometric quantitation of E and F (Panel 1).

in NG and CHG were equal, drawing similarity to the EMSA binding data. These results confirm that CHG-cultured U937 cells are primed to respond faster to the inflammatory cytokine TNFα compared with NG cultured cells.

To further elucidate the mechanism of NF-κB activation, the levels of the inhibitory IκB subunits (IκBa and IκBe) were checked in cytosolic and nuclear extracts under basal conditions and following TNFα challenge in NG and CHG. TNFα mediates degradation of pre-existing IκBa, releasing the trans-acting NF-κB complex, allowing its translocation to the nucleus (37). In addition, the re-synthesized IκBa translocates to the nucleus, binding to the p50/p65 complex, thereby exposing the nuclear export signal for the removal of the latter out of the nucleus (37). We therefore examined re-synthesized levels of IκBa in the cytosol of NG- versus CHG-treated U937 cells following 60–120 min of continuous TNFα treatment. The re-synthesized levels of IκBa in NG were significantly higher (2.87 ± 0.78-fold) compared with its CHG counterpart (Fig. 5, C versus D, panel 3, fifth and sixth lanes). The Western blots of the cytosolic extracts from the time course study were also sequentially probed with anti-IκBa and anti-IκBe antibodies with intermediate stripping. We report here for the first time that the level of IκBa in the cytosol of NG-cultured cells was significantly higher (2.65 ± 0.53-fold) compared with that observed in CHG (Fig. 5, E versus F, panel 1, first lane). Re-synthesized levels of IκBa in the cytosol following 60 or 120 min of TNFα treatment were also higher in NG versus CHG cells (1.85 ± 0.71-fold, Fig. 5, E versus F, panel 1, sixth lane), lending additional support to the higher basal p65 levels in CHG-cultured cells. In contrast to IκBa levels, IκBe levels in IκBβ (data not shown) and IκBe were not affected at any of the time points evaluated (Fig. 5, E versus F, panel 2).

Data from the time course study indicated that U937 cells cultured in CHG are primed for faster response to TNFα and show a stronger rapid activation of NF-κB compared with NG cells. The levels of IκBa were higher in the cytosol of NG versus CHG cells, suggesting a possible mechanism for the higher basal levels of p65 in CHG, which could further explain the increased DNA binding and luciferase activity.

**Effects of Antioxidants on CHG-induced NF-κB Activation**

U937 cells were incubated with antioxidants NAC (100 μM) and PDTC (50 μM) for 1 h before culturing them in CHG for 24 h in the presence of the inhibitors, following which nuclear extracts were made for EMSA. The antioxidants showed striking inhibition of CHG-induced NF-κB binding by blocking the translocation of the p65 subunit to the nucleus (Fig. 6, A and B, third lane versus eighth lane). In addition, the antioxidants also partially blocked TNFα-induced NF-κB activation in NG and CHG (Fig. 6, A and B, second and fourth lanes versus fifth and sixth lanes). PhosphorImager data from multiple EMSA are tabulated in Table III.

**Activation of AP-1 by High Glucose but Not TNFα**

Mutational analysis of the TNFα promoter confirmed a cooperative role of AP-1 with NF-κB in CHG-mediated 295TNF-Fluc activity. The U937 nuclear extracts from the time course study (Fig. 5, A and B) were used to perform EMSA with a 17-bp AP-1 oligonucleotide encompassing the AP-1 site from the proximal TNFα promoter (“Materials and Methods”). AP-1 activation was dramatically stimulated by CHG over NG (Fig. 7A, first lane versus seventh lane). TNFα treatment showed a rapid increase in AP-1 activation in NG (Fig. 7A, first lane versus second through sixth lanes). However, in CHG cells TNFα down-regulated AP-1 activation compared with that observed in CHG alone (Fig. 7A, seventh lane versus ninth or
Mechanism of CHG-induced Monocyte Activation

Pharmacological inhibitors were used to further evaluate the mechanism of CHG-induced monocyte activation. Antioxidants, a p38 MAPK inhibitor, and PKC inhibitors were used to block each of the activated pathways so far identified to be important in monocyte activation, and the counter effects of each inhibitor were evaluated separately or in combination. In our study, CHG induced oxidant stress (higher $O_2^\cdot$) in monocytes and increased phosphorylation of oxidant stress-sensitive MAPKs (p38 and JNK-1). The antioxidants NAC and PDTC had similar counter effects on CHG- or TNF-α-induced NF-κB binding (Table III) and 295TNFpLuc activity (Table IV) or CHG-induced TNFα secretion (Table V). The inhibitory effects of PDTC, known to function as an antioxidant and a NF-κB inhibitor, were slightly greater than that seen with NAC. These results suggest that CHG- and TNF-α-induced monocyte activation could be mediated at least in part by ROS. Since the antioxidants were 60–70% effective, ROS-independent pathways must also contribute to monocyte activation by CHG and TNFα.

We show here for the first time the inhibitory effects of the...
In this study we demonstrated that CHG induced increased oxidant stress in monocytic cells by stimulating elevated levels of \( \text{O}_2^{=} \) (ROS) and increased phosphorylation of stress-responsive MAPKs, p38, and JNK-1. Such oxidant stress incited monocyte activation, measured by elevated TNF\( \alpha \) secretion. We have presented evidence that CHG culturing of monocytic cell lines or normal human monocytes caused a dramatic increase in the release of the inflammatory cytokine TNF\( \alpha \), in part to enhanced TNF\( \alpha \) mRNA transcription. Furthermore, the transcriptional activation was mediated in part by ROS via activation of NF-\( \kappa \)B and AP-1, as suggested by a number of observations. A 295-base pair fragment immediately upstream of the proximal TNF\( \alpha \) promoter containing canonical NF-\( \kappa \)B and AP-1 binding sites reproduced the effect of CHG on TNF\( \alpha \) transcription in a luciferase reporter assay. Mutational analyses of the NF-\( \kappa \)B and AP-1 sites showed cooperativity of the two transcription factors in regulating 295TNF\( \alpha \)Luc activity. Gel shift and supershift analysis using these binding sites showed activation of both the p65 and p50 subunits of NF-\( \kappa \)B and the c-Fos and c-Jun subunits of AP-1 in nuclear extracts of CHG lysates. Western blot analyses demonstrated elevated nuclear levels of p65 and p50 and decreased cytosolic levels of I\( \kappa \)B\( \alpha \) in the CHG-treated monocytes. The contention that ROS act as a key intermediate in this pathway was also supported by several lines of evidence. CHG increased \( O_2^{=} \) levels comparable with that found with TNF\( \alpha \) or PMA. We show evidence here for the first time that CHG stimulated phosphorylation of oxidant stress-sensitive p38 and JNK-1 MAPKs in monocytes. More importantly, antioxidants and p38 inhibitors partially counteracted the effects of CHG on TNF\( \alpha \) production, the 295TNF\( \alpha \)Luc reporter activity, activation of NF-\( \kappa \)B, and repression of I\( \kappa \)B\( \alpha \). The p38 inhibitor blocked monocyte activation by 60%. A combination of the p38 and PKC inhibitors or the PDTC and PKC inhibitors blocked monocyte activation more than 90%. Our study, using pharmacological inhibitors, further suggested that CHG activated monocytes by ROS-dependent and -independent pathways. CHG-mediated monocyte activation involved upstream kinases such as PKC and MAPKs, affecting increased transcription of TNF\( \alpha \) via coordinate activation of downstream transcription factors NF-\( \kappa \)B and AP-1.

We used U937 and THP-1 cells in our study, since these cells have been routinely used to replace human monocytes, the latter of which is difficult to obtain in highly pure and unactivated state. Monocyte activation was not affected by hyperosmolarity of CHG over NG, since adding mannitol to NG culture media did not cause monocyte activation. Our results also suggested that glucose metabolism was essential for monocyte activation, since analogues of glucose, which are not metabolized, such as 3-O-methyl glucose (nonphosphorylated but transported) or 2-deoxyglucose (phosphorylated but not metabolized or transported) did not activate monocytes over NG controls.

Hyperglycemia causes altered redox changes by altering the NADH/NAD\( ^+ \) ratio in the cells, leading to the activation of key inflammatory signals and gene expression (1, 4, 12). Studies in several diabetic microvascular and macrovascular models demonstrate the role of oxidant stress leading to diabetic complications (3). Monocyte cells are capable of \( O_2^{=} \) production in response to a variety of stimuli (6). Our data using monocytic cell lines demonstrated that CHG-mediated \( O_2^{=} \) release was equivalent to TNF\( \alpha \)- or PMA-stimulated \( O_2^{=} \) release in NG, indicating that CHG generated ROS as potently as the inflammatory cytokine or the phorbol ester. The role of oxygen radicals as second messengers for inducing expression of various inflammatory genes in monocyte (macrophage chemotactic protein 1 (MCP-1) and colony stimulating factor-1 (CSF-1)) was reported recently (45). Naturally occurring cellular factors such as glutathione (GSH) are important in maintaining the redox potential in cells and in mounting a defense against oxidant stress in a thiol-sensitive fashion (4). We therefore used two antioxidants particularly effective against intracellular thiol levels, NAC and PDTC, to block monocyte activation. In addition to its role as an antioxidant, PDTC is also an inhibitor of NF-\( \kappa \)B activation. The antioxidants, when present before and during culturing of monocytes in CHG demonstrated significant but partial counter effect on NF-\( \kappa \)B activation, 295TNF\( \alpha \)Luc activity, and TNF\( \alpha \) secretion. PDTC showed additional blockade of all aspects of monocyte activation, strengthening the notion that NF-\( \kappa \)B is important in monocyte activation by CHG. Our results therefore suggest that both ROS-dependent...
and -independent pathways are important in monocyte activation by CHG.

This study provides evidence for the first time for increased phosphorylation of p38 and JNK-1 stress-responsive MAPKs by CHG in monocytes. Basal levels of p38 and pJNK-1 were higher in CHG-cultured THP-1 and U937 cells. Since CHG stimulated release of low levels of TNFα into the conditioned media, we evaluated if the monocyctic cells cultured in CHG were responsive to further stimulation with TNFα. There was a strong overall increase in the levels of pp38 and pJNK-1 in CHG-cultured THP-1 cells, confirming that CHG did not blunt the TNFα responsiveness for MAPK activation in these cells. The overall levels of pp38 or pJNK-1 were higher in CHG over NG following TNFα treatment. CHG and TNFα could potentially use more than one pathway for activation of these MAPKs. Both p38 and JNK-1 are activated by oxidative stress (21), and since CHG culturing significantly increased higher O2 levels over NG, it may be logical to suggest that oxidative stress (ROS) generated by CHG could at least in part account for the observed activation of these two MAPKs in the monocyctic cells. Glucose- and TNFα-induced p38 activation was recently reported in rat aortic smooth muscle cells (19, 20, 22). In our study, TNFα stimulated similar levels of O2 in NG or CHG cells, but activation of MAPKs was additive following TNFα treatment in CHG. Therefore increased phosphorylation of p38 or JNK-1 could only be partially accounted for by CHG-stimulated ROS-dependent pathways. The antioxidant PDTC showed a stronger inhibitory effect on monocyte activation in CHG plus TNFα compared with CHG alone or NG plus TNFα. The p38 inhibitor showed similar counter effects on monocyte activation in CHG alone, NG plus TNFα, or CHG plus TNFα. Therefore, it may be reasonable to conclude that more than one pathway is involved in CHG- or TNFα-mediated p38 activation. However the counter effect of p38 inhibitor on monocyte activation suggested that downstream of p38, CHG or TNFα possibly use similar signals for monocyte activation.

CHG-stimulated ROS and MAPKs were only partially responsible for monocyte activation (inhibitor data), and activation of PKC by high glucose is well established (25, 44). PKC is a known activator of NF-κB (37). We evaluated the importance of the PKC pathway in CHG- and/or TNFα-mediated monocyte activation. PKC activation has been documented both by ROS-dependent and -independent pathways (7, 13, 26). We evaluated the counter effect of PKC inhibitor GF109203X on monocyte activation. The PKC inhibitor blocked different aspects of monocyte activation between 60–65%, similar to the p38 inhibitor, and the inhibition profile was very similar in CHG or with TNFα. Interestingly, GFX in combination with p38 MAPK inhibitor or GFX in combination with PDTC greatly blocked monocyte activation (~90%), confirming the multilevel regulation of the activation process by oxidative stress and/or inflammatory cytokine. Further evidence in support of our finding was recently reported in smooth muscle cells (19, 20) and kidney mesangial cells (24), showing glucose-mediated activation of PKC and p38.

The pleiotropic NF-κB/Rel family of transcription factor has been implicated in a wide variety of inflammatory response (34, 35, 38). The transacting NF-κB complex, stimulated by high glucose or TNFα, was similar, composed of p65/p50 heterodimer. TNFα stimulation of monocyctic cells showed an early activation phase (5–15 min) and a late activation phase (>30 min), which was possibly PKC-dependent. Furthermore, the roles of p38 and PKC in activation of NF-κB has been well documented (19, 22). In U937 cells, the late phase (30–120 min) of NF-κB activation following TNFα treatment was similar in NG and CHG, suggesting that CHG culturing possibly depleted PKC pools, commonly used by both CHG and TNFα for the late phase of NF-κB activation. Nevertheless, the early phase of NF-κB activation was stronger; the 295TNFluc activity as well as TNFα accumulation in the conditioned media were all greater in CHG cells following treatment with TNFα. These results suggest that monocytic cells cultured in CHG were responsive to further challenge with inflammatory cytokines, supporting the vicious inflammatory potential of CHG.

A new finding of our study was the potential mechanism for the CHG-stimulated NF-κB activation. The basal level of the inhibitory subunit IκBα was reduced by 2-fold in the cytosol of the CHG cells compared with NG. We can therefore rationalize that the higher monocyte activation in CHG versus NG could be attributed to the overall lower levels of the inhibitor subunit (IκBα), resulting in failure to sequester all the transacting NF-κB complex in the cytosol. The conclusion was further strengthened by the fact that CHG cells had 2-fold lower levels of resynthesized IκBα in the cytosol following TNFα treatment, as was the level of IκBα in the nuclear extracts, responsible for the export of the transacting complex. This could explain the persistent NF-κB levels in the nucleus, the elevated luciferase activity, and the increased levels of TNFα mRNA and protein observed in CHG-cultured cells. The results suggested a possible impairment in the overall synthesis, specifically of IκBα, since IκBε was not affected.

Another important finding of this study was that CHG-cultured cells were resistant to further AP-1 stimulation by TNFα. Since CHG and AP-1 are both well known activators of PKC, the results indicate that CHG and TNFα may stimulate the same PKC isoforms needed for AP-1 stimulation. TNFα-mediated AP-1 activation could be restored by pretreating the cells with PKC inhibitors but not with the p38 inhibitor SB202190. 2 Examples of such resistance in the activation of kinases or transcription factors were seen in secondary challenges with LPS (34) or in long term challenges with phorbol esters (30). The results from the mutational analysis of the NF-κB3 and AP-1 sites in the 295TNFluc promoter confirmed that NF-κB is the major transcription factor involved in regulating TNFα promoter activity, but maximal transcriptional regulation is achieved by the concerted effort of NF-κB and AP-1 in monocytes.

Using a combination of cellular, biochemical, and molecular assays, we have demonstrated the involvement of ROS and MAPKs in monocyte activation and transcriptional regulation of inflammatory gene expression by CHG, simulating a diabetic state. We have also elucidated a possible mechanism for constitutive NF-κB activation in CHG by demonstrating for the first time lower basal levels of inhibitor IκBα in CHG-cultured U937. The levels of TNFα secreted in CHG could stimulate further production of this cytokine by autocrine control. It is

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2 M. Guha, W. Bai, J. L. Nadler, and R. Natarajan, unpublished observation.
therefore tempting to propose a simple model (Fig. 8) for monocyte activation in hyperglycemia.

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