Molecular Mechanisms of High Glucose–Induced Cyclooxygenase-2 Expression in Monocytes

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The cyclooxygenase (COX)-2 enzyme has been implicated in the pathogenesis of several inflammatory diseases. However, its role in diabetic vascular disease is unclear. In this study, we evaluated the hypothesis that diabetic conditions can induce COX-2 in monocytes. High glucose treatment of THP-1 monocytic cells led to a significant three- to fivefold induction of COX-2 mRNA and protein expression but not COX-1 mRNA. High glucose–induced COX-2 mRNA was blocked by inhibitors of nuclear factor-κB (NF-κB), protein kinase C, and p38 mitogen-activated protein kinase. In addition, an antioxidant and inhibitors of mitochondrial superoxide, NADPH oxidase, and glucose metabolism to glucosamine also blocked high glucose–induced COX-2 expression to varying degrees. High glucose significantly increased transcription from a human COX-2 promoter-luciferase construct (twofold, $P < 0.001$). Promoter deletion analyses and inhibition of transcription by NF-κB superrepressor and cAMP-responsive element binding (CREB) mutants confirmed the involvement of NF-κB and CREB transcription factors in high glucose–induced COX-2 regulation. In addition, isolated peripheral blood monocytes from type 1 and type 2 diabetic patients had high levels of COX-2 mRNA, whereas those from normal volunteers showed no expression. These results show that high glucose and diabetes can augment inflammatory responses by upregulating COX-2 via multiple signaling pathways, leading to monocyte activation relevant to the pathogenesis of diabetes complications. *Diabetes* 53:795–802, 2004

Hyperglycemia has been implicated as a major contributor to several diabetes complications (1,2). Certain key factors have been implicated in diabetes-induced accelerated atherosclerotic and inflammatory disease, including oxidant stress (3,4), formation of advanced glycation end products (AGEs)

(5,6), increased flux through the polyol and hexosamine pathways (4), protein kinase C (PKC) activity (7), and changes in inflammatory gene activities (4,8). Production of mitochondrial superoxide has been implicated as a common factor in these mechanisms of hyperglycemic damage (4,9). Monocyte activation, adhesion to the endothelium, and transmigration into the subendothelial space are key early events in the pathogenesis of atherosclerosis (10). Several inflammatory cytokines and chemokines are implicated in this process. Monocyte-macrophage inflammatory cytokines are also implicated in the pathogenesis of islet destruction in type 1 diabetes (11). We recently demonstrated that high glucose culture of THP-1 monocytes or human peripheral blood monocytes could increase expression of the inflammatory cytokine, tumor necrosis factor-α (TNF-α), and the chemokine, monocyte chemoattractant protein 1 (MCP-1), in an oxidant stress, nuclear factor-κB (NF-κB), and AP-1 transcription factor–dependent manner (8,12). However, although prostaglandins are known to play a role in diabetes complications, very little is known regarding the regulation of formation and action of inflammatory eicosanoid lipids in monocytes under diabetic conditions.

Cyclooxygenases (COX)-1 and -2 catalyze the conversion of arachidonic acid to prostaglandins and related eicosanoids (13–17). COX-1 is constitutively expressed in most cells and plays a role in basal physiological functions in several cells and tissues. COX-2, on the other hand, is usually expressed at low or undetectable levels in most tissues and cells, but is significantly induced by stimuli such as lipopolysaccharide, cytokines such as interleukin (IL)-1α, IL-1β, and TNF-α, and growth factors (13–18). An exception is seen in some tissues (17), including the pancreatic islet that constitutively and dominantly expresses COX-2, (19,20) and where its products, such as prostaglandin E_2 (PGE_2), are believed to play a role in inflammation, islet destruction, and inhibition of insulin secretion (19–22). COX-2 and its products also have renal functions and vascular effects (23,24). They are implicated in the pathogenesis of several inflammatory diseases, and selective inhibition of COX-2 is effective in reversing inflammation without gastric side effects (13,14,17). Although COX-2 can form the vasodilatory and protective prostacyclin, it also produces the potent inflammatory PGE_2 (15–17).

The sequence of the human COX-2 gene is known, and several *cis*-acting regulatory elements have been identified (25). Various stimuli can induce transcription of COX-2 via *cis*-acting elements such as STAT-1, STAT-3, NF-κB, NF-IL6, cAMP-response element (CRE), peroxisome-proliferator–activated receptor–responsive element, and CCAAT/

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enhancer-binding protein transcription factors (25–28). In addition, COX-2 can also be regulated post-transcription-ally (29,30).

COX-2 and its pro-inflammatory products have been implicated in the pathogenesis of atherosclerosis (31,32). COX-2 is also induced by oxidized lipids (27). Since COX-2 inhibitors also block formation of the protective prostacy-clin PGI$_2$, studies have been performed to determine wheth-er these inhibitors could worsen atherosclerosis (24).

High glucose could induce IL-1β in human pancreatic islets (33), but the effects on COX-2 were not examined. A very recent report showed that high glucose increased COX-2 expression in mesangial cells via mitochondrial reactive oxygen species (34). In endothelial cells, high glucose increased COX-2 expression and decreased nitric oxide availability (35). However, very little is known regarding the potential involvement of COX-2 in diabetic vascular complications, diabetic atherosclerosis, or the regulation of COX-2 in relevant cells such as monocytes under diabetic conditions. Interestingly, very recently, COX-2 was shown to be upregulated by ligands of the receptor for AGE (RAGE) in monocytes (36). However, the effects of high glucose on COX-2 in monocytes and the mechanisms involved are not known. This is significant because early cellular dysfunction and inflammatory changes induced by high glucose in monocytes could play a major role in the pathogenesis of diabetes complications. Our studies pro-vide key new information in this connection.

Simulated diabetic conditions in monocytes in vitro, such as high glucose culture or treatment with AGEs, can induce the expression of inflammatory cytokine genes via activation of oxidant stress, specific signaling pathways, and transcription factors such as NF-κB (8,12,36–38). These factors can increase monocyte activation and vas-cular dysfunction associated with diabetes complications. Since many of these cytokines and chemokines regulate COX-2, in the present study we evaluated the hypothesis that high glucose or the diabetic state can increase COX-2 expression and activity, which could then amplify mono-cyte activation and inflammatory responses. We also eval-uated the regulatory and signal transduction mechanisms mediating the effects of high glucose on COX-2 expression.

RESEARCH DESIGN AND METHODS

Actinomycin D, azaserine, apocynin cycloheximide, glucosamine, and thenoyl-trifluoroacetone (TTFA) were from Sigma-Aldrich Chemicals (St. Louis, MO). SB202190, bis-indolylmaleimide (GFx), AG490, and N-acetylcysteine (NAC) were from Calbiochem (San Diego, CA). PD-98059, anti-phospho–cAMP responsive element binding (CREB), and anti-CREB antibodies were from Cell Signaling (Beverly, MA); Bay11-7082 [(E)-3-(4-Methylphenylsulfonyl)-2-pro-penenitrile] from BIOMOL Research Laboratories (Plymouth, PA); and ^{32}P-α-UTP (3,000 Ci/mmol) from Perkin-Elmer Life Sciences. RT-PCR reagents were from Applied Biosystems (Foster City, CA), and RPA III kit and Quantum RNA 18S Internal Standards were from Ambion (Austin, TX). Luciferase assay system was from Promega (Madison, WI). PGE$_2$ enzyme immunosorbent assay (EIA) kit was from Cayman Chemical (Ann Arbor, MI). Anti–COX-2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).
Cell culture and treatments. Human THP-1 monocytic cells were cultured in RPMI-1640 medium as previously described (8,36). For high glucose culture, cells were treated with 15–20 mmol/l D-glucose (high glucose). In some experiments, THP-1 cells were pretreated with actinomycin D (0.1 μg/ml), cycloheximide (0.5 μg/ml), Bay11-7082 (NF-κB inhibitor, 10 μmol/l), AG-490 (Janus tyrosine kinase [JAK] inhibitor, 100 μmol/l), SB202190 (p38 mitogen-activated protein kinase [MAPK] inhibitor, 1 μmol/l), GFx (PKC inhibitor, 0.5 μmol/l), PD-98059 (MEK inhibitor, 25 μmol/l), azaserine (inhib-itor of glutamine: fructose-6-phosphate amidotransferase [GFA], 5 μmol/l),

NAC (antioxidant, 100 μmol/l), apocynin (NADPH oxidase inhibitor, 30 μmol/l), or TTFA (mitochondrial complex II inhibitor that blocks mitochon-drial superoxide production, 10 μmol/l). They were then incubated in normal glucose medium or with high glucose for various time periods.
Isolation of human peripheral blood monocytes. Fifty to sixty milliliters of blood from adults with clinically established type 1 diabetes (three subjects, Pn1, Pn2, and Pn3, with HbA$_{1c}$ levels of 8.0, 8.4, and 6.5%, respectively) or with type 2 diabetes (two subjects, Pn4 and Pn5, with HbA$_{1c}$ levels of 7.5 and 7.4%), or normal healthy donors (four subjects, N1, N2, N3, and N4) were collected in the presence of anti-coagulant. Informed consent was obtained from all participating subjects in accordance with approved IRB guidelines. Mono-cytes were isolated from the blood as previously described (36).
RNA isolation and relative RT-PCR. THP-1 cells (2×10^6/sample) were cultured in medium containing 5.5 (normal glucose) or 20 mmol/l (high glucose) glucose for various time intervals. Total RNA isolation and relative RT-PCRs were performed as previously described (8,36).

The 5′ and 3′ primers for human COX-2 were 5′-ATCTACCCTCCTCAAGT CCC-3′ and 5′-TACCAGAAGGGCAGGATACAG-3′ (product size 708 bp). The 5′ and 3′ primers for COX-1 were 5′-CCGGATGCCAGTCAGGATGATG-3′ and 5′-CTAGACAGCCAGATGCTGACAG-3′ (529 bp). Results are expressed as fold stimulation over normal glucose after normalizing with paired 18S RNA levels. Ribonuclease (RNase) protection assays (RPAs) were performed as previ-ously described (36).
Plasmid construction. Construction of the deletion mutants containing specific regions of the human COX-2 gene promoter in the luciferase reporter vector pGL3 Basic (Promega) was accomplished by PCR amplification. Plasmid hCOX-2 (−1,437/+127) was generated by deleting 5,673 bp DNA fragment from upstream of the recombinant plasmid containing firefly lucif-erase gene under the control of ~7,273-bp promoter region of the hCOX-2 gene (generous gift from Dr. Thomas McIntyre, University of Utah, Salt Lake City) by digesting with restriction enzyme Kpn-I. The resulting plasmid was religated using T4-DNA ligase, yielding the luciferase gene under the control of −1,437/+127 hCOX-2 promoter region. Deletion constructs containing various promoter regions of hCOX-2, namely from −860 to +127, −360 to +127, −218 to +127, −123 to +127, and −52 to +127 were generated using the plasmid containing ~7 kb promoter region of the hCOX-2 gene as a template. The following primers were used: upstream primers, from −860, 5′GGTACCCACATTAACTATTTACAG3′; from −360, 5′GGTACCCCAAGGCG ATCAGTCCAG3′; from −218, 5′GGTACCTACCCCCTCTGCTCCCAA3′; from −123, 5′GGTACCTTTTTTAAGGGGAGAGG3′; from −52, 5′GGTACCCATGG GCTTGGTTTTC3′; and downstream primer +127, 5′AAGCTTCGGGCAGGGC GCGGCGC3′. All upstream PCR primers contained Kpn-I restriction sites (italic), and the downstream primer contained HindIII recognition site (italic), that forced cloning of the fragments in the desired orientation into the pGL3 Basic vector. Orientation and sequence of all constructs were verified by direct sequencing using the ABI PRISM 377 DNA sequencer.
DNA transfections and luciferase assays. THP-1 cells in six-well plates were transfected with 1 μg of the indicated COX-2 promoter plasmids, the control pGL3-Luc plasmid, the pCMV-mIκB plasmid (generous gift from Dr. E. Zandi, University of S. California), or the dominant-negative CREB plasmid, KCREB (39) (generous gift from Dr. Jane E.B. Reusch, University of Colorado) using Lipofectamine 2000, and then luciferase activities determined (8,36).
Data analyses. Data are expressed as mean ± SE of multiple experiments. Paired Student's t tests were used to compare two groups and ANOVA for multiple comparisons. P values <0.05 were considered statistically significant.

RESULTS

High glucose induces COX-2 mRNA but not COX-1 mRNA in THP-1 cells. We initially evaluated whether high glucose could induce COX-2 and COX-1 mRNAs in THP-1 cells. The cells were treated with or without high glucose (15 mmol/l) for various time intervals from 24 to 72 h (Fig. 1). RNA extracted from these experiments was subjected to relative RT-PCR analyses with 18S as internal control. Results show that high glucose treatment mark-edly increased COX-2 mRNA expression by 24 h, which declined by 72 h (Fig. 1*A*). Figure 1*B*, which contains data from multiple experiments normalized to 18S, shows that the effects of high glucose at 24, 48, and 72 h were statis-tically significant. Figure 1*C* shows that high glucose–induced COX-2 mRNA appeared as early as 8 h but not at 4 h. In contrast, COX-1 mRNA was constitutively ex-
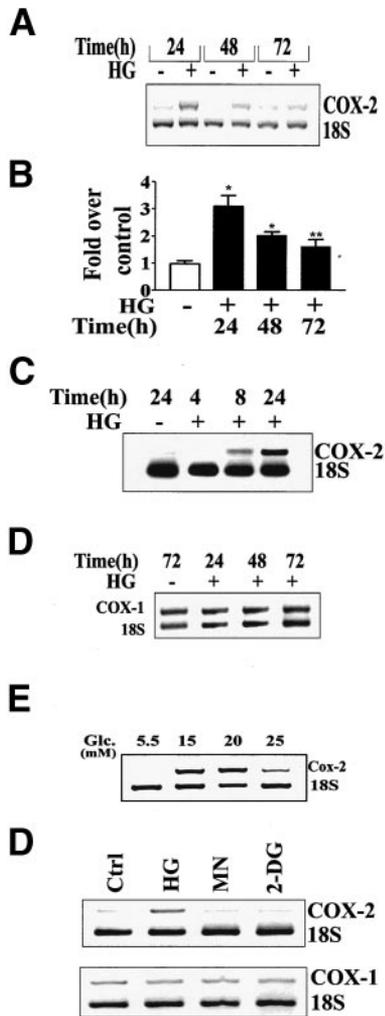
FIG. 1. Analysis of high glucose (HG)–induced COX-2 mRNA expression. Relative RT-PCRs with gene-specific primers were performed with total RNA isolated from THP-1 cells treated with or without high glucose (15 mmol/l) for 24–72 h. − and + symbols above the lanes indicate normal and high glucose–treated cells, respectively. *A*: RT-PCR for COX-2 mRNA. *B*: Data quantitated from 3–5 experiments (*$P <$ 0.001, **$P <$ 0.05 vs. normal glucose). *C*: COX-2 mRNA expression at 4, 8, and 24 h of high glucose. *D*: COX-1 mRNA with same samples. *E*: Dose response effect of glucose on COX-2 mRNA. *F*: Comparison of COX-2 mRNA in cells treated with high glucose (HG), mannitol (MN), or 2-deoxyglucose (2-DG).

pressed and not altered during the 24–72 h time periods (Fig. 1*D*). This suggests that high glucose specifically upregulates the inducible COX-2 isoform and not COX-1. To determine the dose response effects of high glucose, THP-1 cells were treated with different concentrations of high glucose (15, 20, and 25 mmol/l), and COX-2 mRNA levels were determined. Figure 1*E* shows that the maximum effects of high glucose on COX-2 mRNA induction was at 15–20 mmol/l. Furthermore, Fig. 1*F* shows that COX-2 mRNA was increased only by high glucose and not by equimolar concentrations of mannitol (osmolality control) or 2-deoxy glucose (control for glucose metabolism), indicating the specificity of high glucose effects. COX-1 mRNA expression was not altered in any of these conditions (Fig. 1*F*, *lower panel*).

To further confirm the RT-PCR data, we performed sensitive RPAs using ³²P-labeled COX-2 and 18S antisense riboprobes. Figure 2 shows a representative autoradio-

gram. A clear protected COX-2 RNA (254 nt) band was seen in the high glucose (20 mmol/l)-treated sample (*lane 4*), whereas no protected band was seen in the untreated control sample (*lane 3*). The 18S protected bands (internal control) of the predicted sizes are seen in both control and treated lanes. *Lane 1* shows probes used in the experiments, whereas *lane 2* is experiment without RNA. *Lane 5* is labeled molecular weight markers. These results substantiate the RT-PCR data.

COX-2 mRNA is upregulated in monocytes from diabetic individuals. Although THP-1 cells have several properties of monocytes, they may not fully represent peripheral blood monocytes. Hence, to determine the in vivo relevance, we evaluated whether peripheral blood monocytes from diabetic individuals express elevated COX-2 mRNA levels relative to nondiabetic control volunteers. Figure 3*A* shows that monocytes from normal nondiabetic volunteers (N1, N2, N3, and N4) have extremely low and almost insignificant COX-2 mRNA expression levels. However, strong expression of COX-2 mRNA is seen in the three type 1 diabetic patients (Pn1, Pn2, and Pn3) run in duplicate. Furthermore, RNA from two type 2 subjects (Pn4 and Pn5) also show elevated COX-2 mRNA levels relative to the control subjects. Figure 3*B* shows bar graph quantitation of the PCR data after normalization for 18S levels. Interestingly, it appears that COX-2 mRNA levels in the type 2 patients (4.5-fold over control average, last bar on right) are somewhat lower than type 1 patients (31.0 ± 3.5-fold over control subjects) since PCR conditions and RNA amounts were kept identical in all samples. With this limited sample size, there seemed to be no specific correlation with HbA_{1c} levels (see RESEARCH DESIGN AND METHODS). Overall, these results show that diabetic monocytes have high levels of COX-2 expression (20.0 ± 6.6-fold, *third bar*) that may contribute to several inflammatory events.

High glucose induces COX-2 protein expression and activity in THP-1 cells. The RT-PCR and RPA data clearly showed significant induction of COX-2 mRNA by high glucose. We therefore evaluated whether COX-2 protein levels were also regulated. Western blot analysis with specific COX-2 antibody was carried out using total
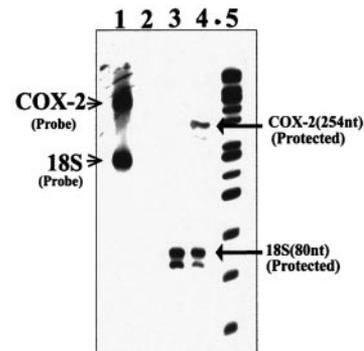

FIG. 2. RNase protection assay to examine COX-2 mRNA induction by high glucose. ³²P-labeled COX-2 (321 nt) and 18S (180 nt) antisense RNAs (50 ng) were allowed to hybridize with 150 µg of total RNA from high glucose–treated and control cells. The RNase protected bands of COX-2 (254 nt) and 18S (80 nt) are indicated. *Lane 1*: ³²P-labeled COX-2 and 18S antisense probes. *Lane 2*: Experiment run without RNA. *Lane 3*: Control. *Lane 4*: High glucose. *Lane 5*: ³²P-labeled molecular weight markers. Arrows show the RNase protected bands. Arrowheads show antisense RNA (probe) of COX-2 and 18S.
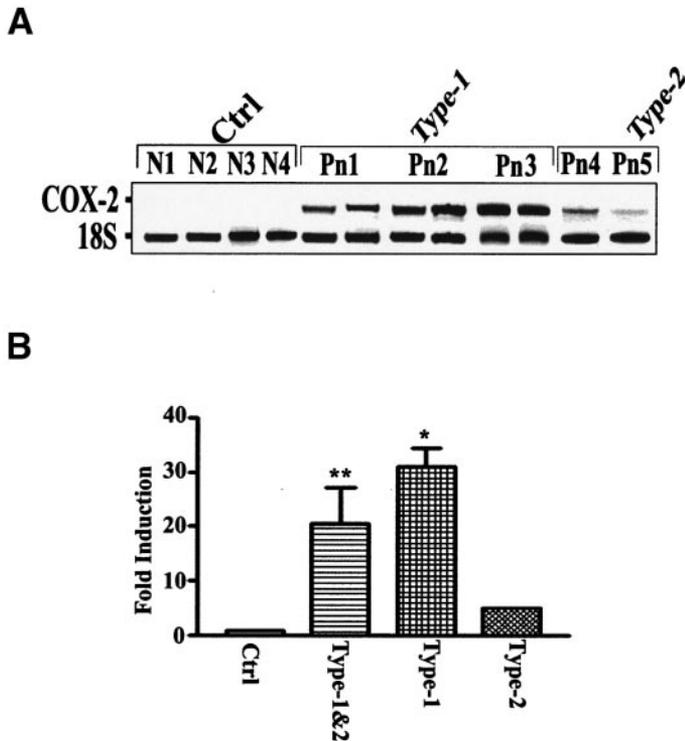
FIG. 3. Monocytes from type 1 and 2 diabetic patients have elevated COX-2 mRNA expression. Peripheral blood monocytes were isolated from three type 1 diabetic patients (Pn1, Pn2, and Pn3), two type 2 diabetic patients (Pn4 and Pn5), or nondiabetic subjects (N1, N2, N3, and N4). The 2×10^5 monocytes were directly processed for RNA isolation. Markedly elevated COX-2 mRNA expression is seen in three type 1 diabetic patients run in duplicate and in two type 2 diabetic patients. In contrast, COX-2 mRNA levels in four nondiabetic subjects were almost undetectable. *A*: PCR data. *B*: Data quantitated from subjects pooled as noted and expressed as fold over normal. *$P <$ 0.001, **$P < 01$ vs. control subjects.

FIG. 4. High glucose (HG) stimulates COX-2 protein expression and PGE_2 formation in THP-1 cells. *A*: High glucose stimulates COX-2 protein levels. Total protein isolated from control THP-1 cells in normal glucose (72 h), and cells treated with high glucose were immunoblotted with anti-COX-2 antibody. *Upper panel*: COX-2. *Lower panel*: The same blot stripped and probed with actin. *B*: High glucose stimulates PGE_2 production. PGE_2 was measured by EIA in culture supernatants of THP-1 cells treated with or without high glucose for 24 h. Data are means ± SE of three independent experiments. *$P <$ 0.001 vs. high glucose. U.D, undetectable.

(Fig. 5). Thus, COX-2 induction, at least at 24 h, is due to increases in transcription. However, further studies are needed to determine the role of de novo protein synthesis at other time periods because COX-2 is known to be regulated transcriptionally and post-transcriptionally via mRNA stabilization.

High glucose treatment activates transcription from the COX-2 promoter. To examine whether high glucose at 24 h can induce transcription from the COX-2 promoter, we made several constructs with luciferase gene under the control of various regions of the hCOX-2 promoter. As shown in Fig. 6*A* and *B*, deletion constructs pCOX-2 (−1,430/+127), pCOX-2 (−860/+127), pCOX-2 (−360/+127), pCOX-2 (−216/+127), pCOX-2 (−123/+127), and pCOX-2 (−52/+127) were transfected into THP-1 cells and then treated with high glucose for 24 h. High glucose could significantly increase luciferase activity in pCOX2 (−360/+127) and pCOX2 (−123/+127) transfected samples (Fig. 6*B*) ($P < 0.001$). However, the longer promoter constructs did not yield significant increase in luciferase activity (Fig. 6*B*). This suggests that high glucose–induced COX-2 up-regulation is mediated by key promoter elements present

protein from control and high glucose–treated THP-1 cells. Figure 4*A* shows that COX-2 protein appeared ~24 h after high glucose treatment and remains sustained up to 72 h (Fig. 4*A, upper panel*). This is consistent with the mRNA data and demonstrates that COX-2 protein and its mRNA peak around similar time points. Equal protein loading was confirmed by probing with an anti-actin antibody (Fig. 4*A, lower panel*). To determine whether the induction of COX-2 mRNA and protein were also associated with increased COX-2 enzyme activity, we examined the levels of the COX-2 product PGE_2. High glucose–treated THP-1 cells had significantly elevated PGE_2 levels relative to normal glucose (410 ± 43 fg/ml, $P < 0.001$) (Fig. 4*B*) as measured by specific EIA.

Increased COX-2 expression by high glucose is predominantly due to transcriptional regulation. Time course analysis showed that high glucose–induced expression of COX-2 mRNA appears at ~8 h, peaks at ~24 h, and then declines by 72 h (Fig. 1). To determine whether high glucose–induced COX-2 mRNA expression at 24 h is due to increases in transcription and/or de novo protein synthesis, THP-1 cells were pretreated for 1 h with actinomycin-D, an inhibitor of transcription, or cycloheximide, a protein synthesis inhibitor, and then treated with high glucose for 24 h. RT-PCR analyses showed that although actinomycin D completely blocked high glucose–induced COX-2 mRNA expression; cycloheximide did not block it
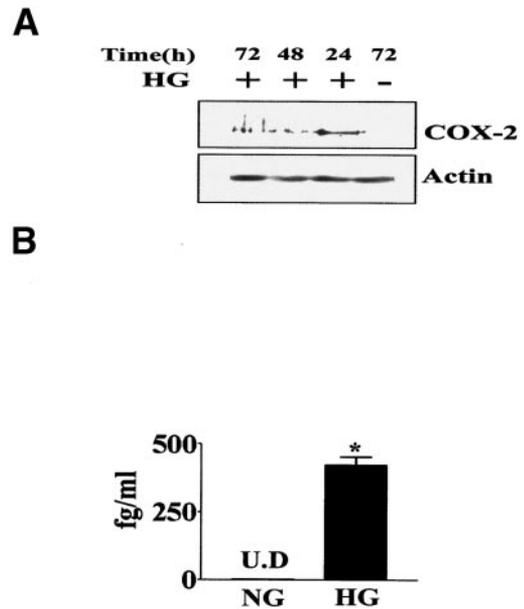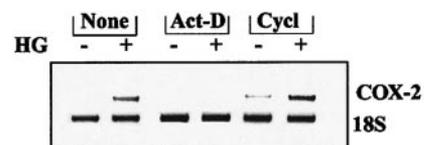

FIG. 5. High glucose (HG) treatment induces COX-2 mRNA by increasing transcription. THP-1 cells were pretreated with actinomycin D (0.1ug/ml) (Act-D) or cycloheximide (0.5ug/ml) (Cycl) and then stimulated with high glucose for 24 h. Relative RT-PCRs with gene-specific primers were performed with total RNA isolated from THP-1 cells treated with or without high glucose (15 mmol/l) for 24–72 h.
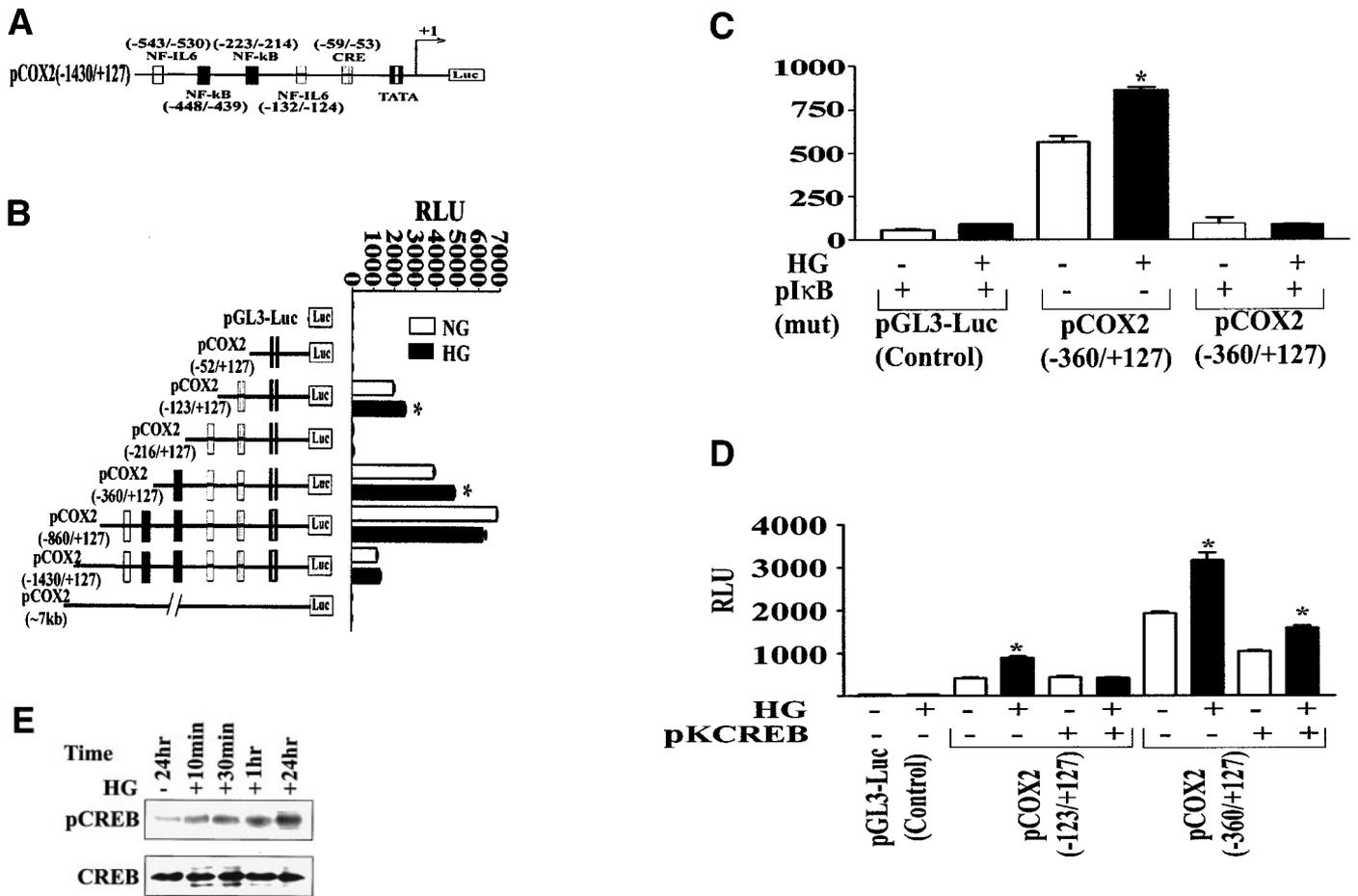
FIG. 6. High glucose (HG) stimulates transcription from the COX-2 promoter in THP-1 cells. Involvement of specific NF-kB and CRE sites. The promoter activities of a series of 5′ deletion mutants made in the COX-2 promoter flanking region were analyzed by transient transfection into THP-1 cells followed by treatment with or without high glucose (20 mmol/l). A: Indication of the TATA box (TATA) and several enhancer sites. B: Luciferase activities of several COX-2 promoter deletion constructs. The −360 and −123 flanking regions are responsive to high glucose. Data are fold luciferase activities over respective control as means ± SE of three independent experiments (*P < 0.001 vs. control). C: Involvement of NF-κB in high glucose–stimulated COX-2 promoter (pCOX-2 −360/+127) activation. Cotransfection of an IκB mutant plasmid along with the −360 deletion mutant showed significant inhibition in high glucose–induced activity. This, along with the observed increase in transcriptional activity in the −360 regions indicates the importance of the proximal NF-κB (at −223/−214) in high glucose effects. D: Involvement of CREB in high glucose stimulated COX-2 promoter (pCOX-2 −123/+127) activation. Cotransfection of CREB mutant plasmid along with the −360 and −123 deletion mutants showed significant inhibition in high glucose–induced COX-2 promoter activation. This, along with the observed increase in transcriptional activity in −360 and −123 flanking regions indicates the importance of CREB site (−59/−53). E: Western blot shows high glucose–induced phosphorylation of CREB in THP-1 cells. Upper panel: Phospho-CREB. Lower panel: Total CREB. NG, normal glucose; RLU, relative light unit.

within the 360-bp promoter region, but certain repressive elements could be present more upstream.

The −360 region of the hCOX-2 promoter (−360/+127) contains several *cis*-acting elements. We were interested in the potential involvement of two *cis*-acting elements near the 5′ end of the coding region, namely NF-κB and CRE binding sites (Fig. 6A), both of which have been implicated in COX-2 gene transcription by various agonists. We first explored NF-κB involvement by cotransfecting THP-1 cells with phCOX2 (−360/+127) and a NF-κB super-suppressor IκB (mut) plasmid that suppresses NF-κB activation. Figure 6C shows that mutant IκB significantly inhibited high glucose–induced COX-2 promoter activity but did not alter control pGL3-Luc. This indicates involvement of the proximal NF-κB site within the −360 region in the high glucose response.

Since significant increase in reporter gene luciferase activity was also observed in the −123/+127 construct that has a CRE site, we examined the potential involvement of CREB transcription factor. This was done by cotransfection of pCOX2 (−123/+127) or pCOX2 (−360/+127) constructs with a mutant CREB plasmid (KCREB) that inhibits the action of CREB by forming heterodimers with the endogenous protein that are incapable of binding target DNA sequences (39). Figure 6D shows that KCREB completely abrogated luciferase activity induced by the −123/+127 construct and partially blocked activity with the −360/+127 construct, thus suggesting involvement of CREB binding to CRE elements in high glucose–induced COX-2 expression. To further validate this, we examined whether high glucose can increase CREB activation. The Western blot in Fig. 6E shows that high glucose led to a marked time-dependent increase in the phosphorylation of CREB at Ser 133, which is known to increase the transcriptional activity of CREB. These results suggest that high glucose–induced COX-2 regulation can be mediated by not only NF-κB but also by CREB.

Signal transduction mechanisms involved in high glucose–induced COX-2 mRNA expression. To determine the key signal transduction pathways involved in high
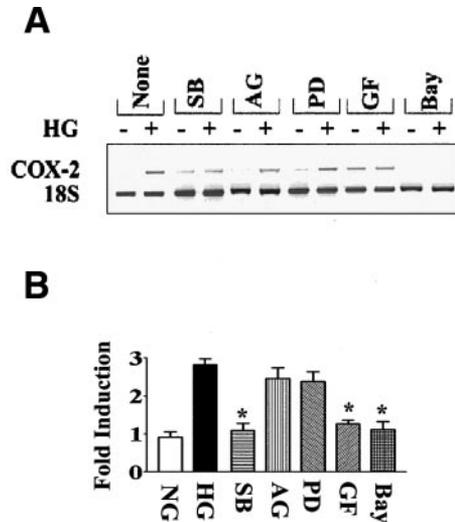
FIG. 7. Effect of signal transducing kinase inhibitors on high glucose (HG)–induced COX-2 mRNA. THP-1 cells were pretreated with inhibitors as shown for 1 h before high glucose stimulation, and then COX-2 mRNA levels were analyzed. *A*: Representative gel of COX-2 and 18S PCR products. *B*: Data quantitated from 3–4 experiments expressed as fold over respective control. *$P < 0.001$ vs. high glucose. NG, normal glucose.

glucose–induced COX-2 mRNA, we evaluated the effects of inhibitors of pathways known to be activated by high glucose, including signaling kinases and oxidant stress. We pretreated THP-1 cells with SB202190 (SB, p38MAPK inhibitor), AG-490 (AG, JAK inhibitor), PD98059 (PD, extracellular signal–related kinase (ERK) 1/2 MAPK inhibitor), GFx (GF, PKC inhibitor), or Bay 11-7082 (Bay, NF-κB inhibitor) and then treated with high glucose for 24 h. Results (Fig. 7*A* and *B*) show that high glucose–induced COX-2 mRNA expression was significantly blocked by inhibitors of p38MAPK, PKC, and NF-κB, but not JAK or ERK1/2 MAPK (Fig. 7*B*). These results implicate the involvement of multiple pathways, including the p38 MAPK, PKC, and NF-κB in high glucose–induced COX-2 mRNA expression.

Effect of antioxidants and metabolic inhibitors on high glucose–induced COX-2 mRNA. Excess glucose may exert effects via metabolism through the hexosamine biosynthesis pathway by GFA activation and glucosamine formation (40). Hyperglycemia can also increase mitochondrial superoxide production (4,9). In monocytes, high glucose–induced superoxide generation also involves NADPH oxidase (8,41). To elucidate the role of some of these pathways in high glucose–induced COX-2 mRNA expression, cells were preincubated with an antioxidant (NAC), NADPH oxidase inhibitor (apocynin), mitochondrial complex-II inhibitor that blocks superoxide production (TTFA), and GFA inhibitor (azaserine). The representative RT-PCR blot in Fig. 8*A* and the bar graph quantitation in Fig. 8*B* show that all four inhibitors significantly blocked high glucose–induced COX-2 mRNA expression in THP-1 cells. Interestingly, direct treatment of THP-1 cells with glucosamine for 24 h could also directly induce COX-2 expression (data not shown), further supporting the idea that glucose metabolism is involved in high glucose-stimulated COX-2 induction.

DISCUSSION

COX-2 and its products, such as PGE$_2$, have been implicated in several inflammatory diseases such as atherosclerosis and in the inhibition of insulin secretion and islet dysfunction related to type 1 diabetes (19–22,31,32). Although changes in vascular prostaglandin production are implicated in vascular reactivity derangements associated with diabetes (42), the role of COX-2 in diabetic monocytes and atherosclerosis is not very clear. RAGE ligation can stimulate COX-2 expression, which can induce monocyte activation (36). In the present study, we demonstrated for the first time that treatment of monocytes with high glucose can significantly increase COX-2 expression and activity in human monocytes. In contrast, high glucose did not alter the COX-1 isoform. High glucose–induced COX-2 expression was specific, since mannitol and 2-deoxyglucose had no effect. Furthermore, in vivo relevance to diabetes complications was suggested by our observation that high levels of COX-2 mRNA are present in monocytes from type 1 or type 2 diabetic patients, but not from normal volunteers.

We also demonstrated that high glucose–induced COX-2 mRNA expression at 24 h was transcriptionally regulated because actinomycin D blocked expression; furthermore, high glucose could increase transcription from a minimal human COX-2 promoter luciferase construct. Promoter deletion analysis and use of pharmacological and genetic inhibitors of NF-κB demonstrated the essential role of NF-κB in high glucose–induced COX-2 gene transcription. The human COX-2 promoter has two NF-κB consensus sites (25), one located within −455 to −428 bases and the other within −232 to −205 bases from the transcriptional start site. We noted that high glucose response was confined to a region within the 360 bp upstream of the 5′ end, indicating that the proximal NF-κB site (−232 to −205) is essential for high glucose–induced COX-2 transcription. Interestingly, this differs from the actions of the RAGE ligand, S100b, that seemed to induce COX-2 via the distal NF-κB site (−455 to −428). Another key difference between the actions of S100b and high glucose is our
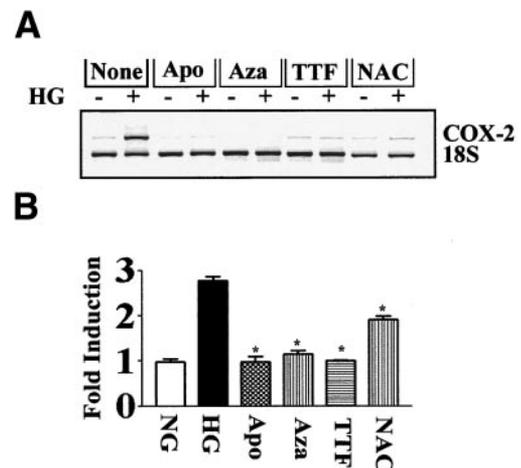

FIG. 8. Effects of antioxidants, inhibitors of superoxide, and metabolic inhibitors. THP-1 cells were pretreated with or without the indicated inhibitors in normal glucose (NG) or high glucose (HG) medium for 24 h and then COX-2 mRNA quantitated. *A*: Representative blot. *B*: Data from 3–4 experiments expressed as fold over respective control (*$P < 0.001$ vs. high glucose).

present observation for the first time that high glucose–induced COX-2 also involves the proximal CRE site and activation of CREB. Whereas reports have shown the effects on high glucose on CREB in vascular smooth muscle cells (43) and mesangial cells (44), our results suggest a novel new role for CREB in monocytes. Thus, whereas both AGEs and high glucose can induce COX-2 expression in monocytes, they differ in the molecular mechanisms of regulation.

We also evaluated the signaling mechanisms involved in high glucose–induced COX-2 mRNA. Using pathway-specific inhibitors, we noted that activation of NF-κB, PKC, and p38 MAPK were involved in high glucose–induced COX-2 mRNA. This indicates the operation of multiple related pathways in COX-2 regulation in monocytes under diabetic conditions. In contrast, the JAK-STAT and ERK pathways did not seem to be involved.

In addition, high glucose–induced COX-2 expression was blocked by NAC, azaserine apocynin, and TTFA, thus suggesting the involvement of oxidant stress, glucosamine production, NADPH oxidase, and mitochondrial superoxide, respectively. These are related pathways since mitochondrial superoxide ($O_2^-\cdot$) production has been implicated in the activation of several of these downstream pathways by high glucose including PKC (4). NADPH oxidase and PKC-α have also been implicated in monocytic $O_2^-\cdot$ release (41,45). Furthermore, high glucose–induced expression of the inflammatory chemokine MCP-1 in monocytes was regulated by both mitochondrial superoxide and NAPDH oxidase (8). Thus, in monocytes, high glucose–induced $O_2^-\cdot$ release may be via both NADPH oxidase and mitochondrial sources. This increased $O_2^-\cdot$ may trigger the activation of NF-κB to induce proinflammatory genes such as COX-2 in diabetic patients.

Some of the downstream consequences of high glucose effects are mediated by the hexosamine biosynthesis pathway, in which fructose-6-phosphate is converted to glucosamine-6-phosphate by rate limiting enzyme GFA (40). Glucosamine can directly induce growth factor gene expression and simulate several glucose effects (44,46). Evidence shows that high glucose and glucosamine-induced PKC and cAMP-dependent protein kinase signaling pathways may participate in hexosamine-induced fibronectin synthesis via CREB phosphorylation and nuclear CREB regulated gene expression via CRE elements (44). In our studies, high glucose–induced COX-2 mRNA was inhibited by a GFA inhibitor azaserine, suggesting the involvement of the hexosamine pathway. Furthermore, our unpublished results indicate that glucosamine can also increase COX-2 mRNA expression in THP-1 cells. Interestingly, since we demonstrated the involvement of a CRE site in the proximal COX-2 promoter, and high glucose also phosphorylated CREB, it is possible that high glucose conversion to glucosamine contributes to COX-2 induction via CREB activation.

In certain cells COX-2 induction is due to increase in post-transcriptional mRNA stabilization rather than increased transcription (29,30). This has been attributed to the presence of several adenylate uridylate–rich elements in the 3′ untranslated region of the COX-2 mRNA. Several mRNA stabilizing proteins have been shown to bind to this region (29,30,47,48). In our studies, it appears that high glucose–induced COX-2 mRNA at 24 h is primarily regulated transcriptionally. However, since COX-2 mRNA is seen at even ~8 h, some of the early effects of high glucose may be partly mediated by mRNA stabilization. Additional studies are needed to evaluate this.

Evidence shows that high glucose culture of monocytes can lead to the transcriptional regulation of TNF-α and MCP-1 via oxidant stress-dependent key signaling mechanisms and NF-κB (8,12). High glucose culture of THP-1 monocytes also significantly increased their adherence to human aortic endothelial cells (8). Very recently, gene profiling with DNA arrays demonstrated that high glucose regulates several inflammatory genes in THP-1 monocytes (8), many of which are regulated by NF-κB. The present data showing the importance of NF-κB in COX-2 regulation by high glucose further underscores the key role played by this transcription factor in diabetes complications. Our results also implicate a new proinflammatory role for CREB in monocytes. Thus the diabetic environment results in the induction of multiple inflammatory mediators by cells in the vessel wall and in circulation, thereby leading to intra- and intercellular activation. Interestingly a very recent report demonstrated enhanced inflammatory reactions and COX-2 expression in human diabetic atherosclerotic plaque macrophages (49). Thus specific COX-2 inhibitors might be beneficial for diabetes complications. Induction of COX-2 by high glucose suggests that eicosanoid products, apart from their vasoactive roles, can also amplify monocyte activation and related diabetes complications.

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