

Activation of Nuclear Factor- κ B by Hyperglycemia in Vascular Smooth Muscle Cells Is Regulated by Aldose Reductase

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Activation of the polyol pathway has been linked to the development of secondary diabetic complications. However, the underlying molecular mechanisms remain unclear. To probe the contribution of this pathway, we examined whether inhibition of aldose reductase, which catalyzes the first step of the pathway, affects hyperglycemia-induced activation of the inflammatory transcription factor nuclear factor (NF)- κ B. Treatment of vascular smooth muscle cells with the aldose reductase inhibitors tolrestat and sorbinil prevented high-glucose-induced protein kinase C (PKC) activation, nuclear translocation of NF- κ B, phosphorylation of IKK, and the increase in the expression of intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and aldose reductase. High-glucose-induced NF- κ B activation was also prevented by the PKC inhibitors chelerythrine and calphostin C. Ablation of aldose reductase by small interference RNA (siRNA) prevented high-glucose-induced NF- κ B and AP-1 activation but did not affect the activity of SP-1 or OCT-1. Stimulation with iso-osmotic mannitol activated NF- κ B and increased the expression of aldose reductase but not ICAM-1 and VCAM-1. Treatment with aldose reductase inhibitors or aldose reductase siRNA did not affect mannitol-induced NF- κ B or AP-1 activation. Administration of tolrestat (15 mg \cdot kg⁻¹ \cdot day⁻¹) decreased the abundance of activated NF- κ B in balloon-injured carotid arteries of diabetic rats. Collectively, these results suggest that inhibition of aldose reductase, which prevents PKC-dependent nonosmotic NF- κ B activation, may be a useful approach for treating vascular inflammation caused by diabetes. *Diabetes* 53:2910–2920, 2004

Long-term diabetes is associated with the development of secondary complications that result in nephropathy, neuropathy, and cataract development (1–3). Diabetes is associated with a two- to fourfold higher risk of cardiovascular diseases and

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AGE, advanced glycosylation end product; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility gel shift assay; ICAM, intracellular adhesion molecule; NF, nuclear factor; PKC, protein kinase C; siRNA, small interference RNA; VCAM, vascular cell adhesion molecule; VSMC, vascular smooth muscle cell.

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increased incidence of both macrovascular and microvascular complications (4). Although diabetes and hyperglycemia induce multiple changes in metabolism and signaling (5), previous investigations suggested that chronic inflammation and activation of the transcription factor nuclear factor (NF)- κ B may be a significant feature of secondary diabetic complications. Several cell types, including vascular smooth muscle cells (VSMCs) (6), endothelial cells (7–9), lens epithelial cells (10), and retinal pericytes (11) when cultured in high glucose show increased NF- κ B activity. Activation of NF- κ B has also been reported in cells that were exposed to advanced glycosylation end products (AGEs) or AGE-albumin adducts that are generated during diabetes (9,12–15).

Persistently higher levels of NF- κ B have been reported in the target organs of diabetic rats such as the retina, the heart, and the kidney (16). Moreover, VSMCs cultured in high glucose display pronounced NF- κ B activation in response to tumor necrosis factor- α , platelet-derived growth factor, interleukin-1 β , and epidermal growth factor (6). In human studies, increased NF- κ B has been shown in retinal pericytes (11), proximal tubular epithelial cells excreted in the urine (13), and mononuclear cells of individuals with diabetes (17). Recently, activated NF- κ B was found to be associated with atherosclerotic plaques in patients with type 2 diabetes (18). Together, this evidence supports the view that NF- κ B activation and the attendant inflammatory changes may be critical determinants of diabetic complications. Nevertheless, the metabolic changes that lead to chronic NF- κ B activation in diabetic tissues remain unknown.

Activation of the polyol pathway is one of the key metabolic changes caused by hyperglycemia (3,19). Under euglycemic conditions, the polyol pathway represents a minor source of glucose utilization, accounting for <3% of glucose consumption. However, in the presence of high glucose, the activity of this pathway is substantially increased and could represent up to 30% of total glucose consumption (19). Abnormal activation of the polyol pathway during diabetes leads to depletion of reducing equivalents and accumulation of osmotically active polyols (3,19). These changes induce osmotic and oxidative stress, which result in chronic tissue injury. Consistent with this view, it has been shown that inhibition of aldose reductase prevents the development of diabetic nephropathy, neuropathy, and cataractogenesis (2,19,20). However, the mechanisms by which inhibition of aldose reductase prevents diabetic complications are not well understood.

In the present study, we tested the hypothesis that the protective effects of aldose reductase inhibition are due to inhibition of hyperglycemia-induced NF- κ B activation. Furthermore, to delineate the mechanism by which inhibition of aldose reductase prevents hyperglycemia-induced NF- κ B activation, we examined intracellular signaling events that precede and follow NF- κ B activation. Our results show that inhibition of aldose reductase prevents NF- κ B activation in VSMCs that are exposed to high glucose in culture and in injured diabetic vessels in situ.

RESEARCH DESIGN AND METHODS

PBS, penicillin/streptomycin solution, trypsin, FBS, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Life Technologies. Antibodies against I κ B- α and p65 were obtained from Santa Cruz Biotechnology. Phospho-I κ B- α (Ser³²) antibody was purchased from New England BioLabs. Polyclonal antibodies against phospho-IKK- α (Ser¹⁸⁰)/IKK- β (Ser¹⁸¹) and IKK- α , - β , and - γ were obtained from Cell Signaling Tech. Sorbinil and tolrestat were gifts from Pfizer and American Home Products, respectively. Consensus oligonucleotides for NF- κ B, AP-1, SP-1, and OCT-1 transcription factors were obtained from Promega. All other reagents were of highest purity available.

Cell culture. Rat VSMCs were isolated from healthy rat aorta and characterized by smooth muscle cell-specific α -actin expression. VSMCs were maintained and grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

Electrophoretic mobility gel shift assay. The VSMCs were pretreated with various concentrations of aldose reductase inhibitors for 24 h and then with glucose or mannitol for 3 h at 37°C. The cytosolic as well as nuclear extracts were prepared and electrophoretic mobility gel shift assay (EMSA) was performed as described before (21). The specificity of the assay was examined by competition with excess of unlabeled oligonucleotide and supershift assays with antibodies to p65. The intensities of the radiolabeled bands were quantified by an Alpha Imager 2000 Scanning Densitometer equipped with the AlphaEase Version 3.3b software. Values from three separate experiments for each treatment were used for statistical analysis.

Determination of NF- κ B translocation. After the indicated treatments, the VSMCs were fixed in 100% ice-cold acetone for 5 min and washed with PBS and then were treated with 10% goat serum in PBS for 30 min to block nonspecific binding. Primary antibodies against p65 were diluted 1:500 in 10% goat serum, and the cells were incubated overnight with the diluted antibodies at 4°C. After washing with PBS, the cells were incubated with appropriate Alexa-488 secondary antibodies in 10% goat serum for 1 h at room temperature in the dark. The cells were then washed with PBS and mounted on slides, and a drop of Fluorsave reagent was added. Fluorescence staining was evaluated using Nikon Eclipse E800 epifluorescence microscope connected to a digital camera and interfaced with a computer.

Western analysis. After the indicated treatments, cell extracts were prepared in PBS that contained 25 μ l of protease inhibitor cocktail and was pooled from three independent experimental sets. Aliquots of the cell extract (40–100 μ g of protein) were separated by SDS-PAGE, and Western blot analyses were carried out using the indicated antibodies. Antibody binding was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). For each set of data, a minimum of three different pools of cells were used. Immunopositive bands were quantified by scanning densitometry.

Determination of protein kinase C activity. The protein kinase C (PKC) activity was measured by using the Promega SignaTECT PKC assay system according to the manufacturer's instructions and as described earlier (21). Briefly, aliquots of the reaction (25 mmol/l Tris-HCl [pH 7.5], 1.6 mg/ml phosphatidylserine, 0.16 mg/ml diacylglycerol [DAG]), and 50 mmol/l MgCl₂) were mixed with [γ -³²P]ATP (3,000 Ci/mmol, 10 μ Ci/ μ l) and incubated at 30°C for 10 min. The extent of phosphorylation was detected by measuring radioactivity retained on the paper.

RNA interference ablation of aldose reductase. Small interference RNAs (siRNAs) were designed to target the coding sequence of rat aldose reductase (GenBank accession no. M60322). The target sequences were directed to the single-strand region according to the predicted secondary RNA structure. Sequences of the form (AA/CA)_N with GC content <55% were selected from this region. Two of such 19 nucleotide RNAs followed by TT were designed and then were chemically synthesized, purified, and annealed (Table 1). Control nonsilencing siRNA (fluorescein) was obtained from Qiagen. VSMCs were grown in DMEM that contained 10% FBS and 1% penicillin and streptomycin at 37°C and 5% CO₂ and seeded on 6-well plates or 96-well plates. When the cells reached 60–70% confluence (in 24 h), the medium was replaced

TABLE 1

Target sequences of aldose reductase (AR) used for design of siRNA

| | Target sequence | Position in DNA sequence |
|-----------|-----------------------|--------------------------|
| AR siRNA1 | AAGCAATCGGAGTCTCCAAC | 502 |
| AR siRNA2 | AAAGGAACCTGGTCTGTATCC | 802 |

with fresh DMEM without serum, and the cells were incubated with the siRNA to a final concentration of 100 nmol and the RNAiFect transfection reagent (Qiagen) as per the supplier's instructions. After incubation for 15 min at 25°C, the medium was aspirated and replaced with fresh DMEM that contained 10% serum added drop-wise to the cells. The cells were cultured for 48 and 72 h at 37°C (5% CO₂), and changes in aldose reductase were determined by measuring aldose reductase protein on Western blots using anti-aldose reductase antibodies and by measuring aldose reductase activity in the total cell lysates (22).

Balloon injury of carotid arteries. Sprague-Dawley rats (250–300 g) were made diabetic by a single injection of streptozotocin (55 mg/kg intraperitoneally) as described earlier (23,24). Blood glucose was monitored for up to 2 weeks, and only the rats with blood glucose >300 mg/dl were used for further experiments. Control rats were treated with the vehicle only (blood glucose <100 mg/dl). Four weeks after injection of streptozotocin, the carotid arteries were balloon-injured as described before (21,25). One day before the injury and throughout the observation time, 10 rats in each group were gavage-fed either tolrestat (15 mg \cdot kg⁻¹ \cdot day⁻¹) or vehicle (2.5 mmol/l sodium bicarbonate). The injured and uninjured arteries were perfusion fixed with 4% paraformaldehyde and stored in 70% ethanol. All animal protocols were approved by the institutional animal care committee.

Immunohistochemistry and histology. For measuring changes in the expression of aldose reductase, fixed specimens of carotid arteries were embedded in paraffin, sectioned at 5 μ m, floated on a protein-free water bath, and picked up on positively charged glass slides. The slides were air-dried overnight, oven-dried at 58°C for 1 h, cooled, heat-fixed, dewaxed in xylene, hydrated in alcohol, placed in distilled water, and stained for activated NF- κ B as described previously (21). Briefly, the sections were placed in a pressure cooker in target retrieval solution (Dako catalog no. S1699) that consisted of a citrate buffer (pH 6.0) for 27.5 min. Slides were cooled rapidly and immunostained using the Dako Autostainer. The slides were washed in Tris buffer (Dako catalog no. S1968), and endogenous peroxidase was removed with 3% hydrogen peroxide. The slides were incubated in primary antibody diluted at 1:100 (10 μ g of the primary antibody) for 120 min, further incubated in the detection system (Dako catalog no. K0609), the link, and the label, each for 20 min, and then in the chromogen diaminobenzidine (Dako catalog no. K3466) for 10 min. Nuclei were stained in Mayer's hematoxylin at half strength. Areas of positive reactivity are stained brown.

Statistical analysis. Data are presented as means \pm SE, and the *P* values were determined using the unpaired Student's *t* test.

RESULTS

Inhibition of aldose reductase prevents high-glucose-induced NF- κ B activation. We first examined whether inhibition of aldose reductase prevents high glucose-mediated NF- κ B activation. Upon culturing VSMCs in the presence of 25 mmol/l glucose, pronounced NF- κ B activation was observed (Fig. 1A). For examining the role of aldose reductase, VSMCs were preincubated for 24 h with different concentrations of the aldose reductase inhibitor sorbinil, followed by the addition of glucose (19.5 mmol/l) in the medium with normal glucose (5.5 mmol/l) to achieve a final concentration of 25 mmol/l. Cells were incubated at this glucose concentration for 3 h at 37°C, and NF- κ B activity was measured by EMSA. For ascertaining that the gel-retarded band, observed in the extracts of cells that were treated with high glucose, was indeed due to NF- κ B, nuclear extract from the high glucose-treated cells was incubated with antibodies to p65 subunit and NF- κ B activity was measured again. Antibodies to p65

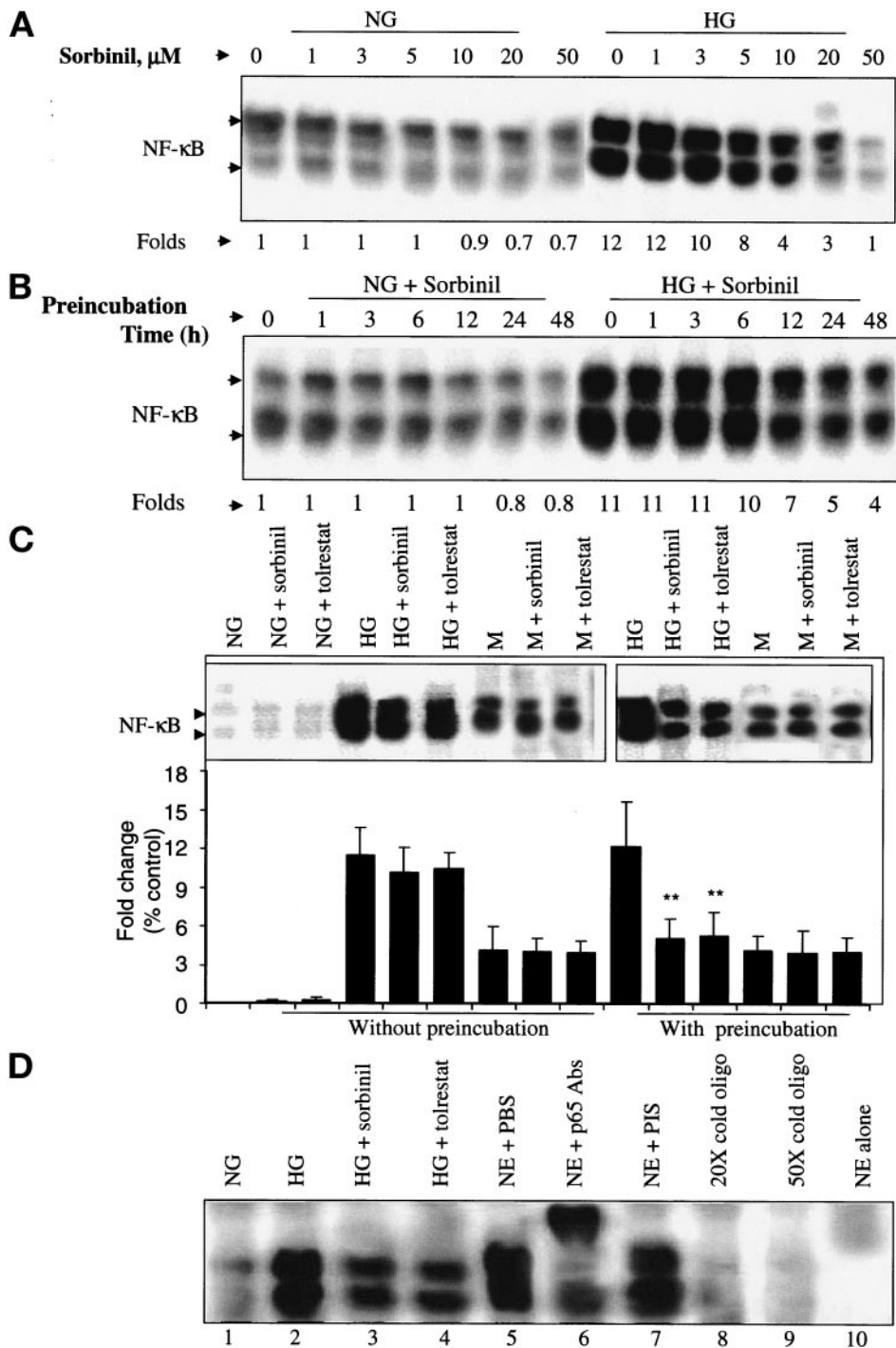


FIG. 1. Inhibition of aldose reductase attenuates high-glucose-induced NF- κB activation. **A:** Concentration-dependent inhibition of NF- κB activation by sorbinil. EMSA autoradiographs of nuclear extracts prepared from serum-starved VSMCs that were treated with 0–50 $\mu\text{mol/l}$ sorbinil for 24 h in either 5.5 mmol/l glucose (normal glucose [NG]) or 25 mmol/l glucose (high glucose [HG]) for 3 h. **B:** Time course of inhibition. EMSA of nuclear extracts from serum-starved VSMCs that were treated with sorbinil (10 $\mu\text{mol/l}$) for the indicated times (shown as “preincubation time” at the top left of the figure) and then stimulated with HG for 3 h. Changes (fold) in NF- κB activity subsequent to various treatments relative to untreated cells are shown at the bottom of the figure. **C:** Growth-arrested VSMCs were treated with 10 $\mu\text{mol/l}$ sorbinil or tolrestat in NG or HG or NG + mannitol (M) (19.5 mmol/l) for 3 h (right, “without preincubation”) or pretreated with aldose reductase inhibitors for 24 h (“with preincubation”) followed by the addition of glucose or M for an additional 3 h. Data represent means \pm SE of three independent analyses; ** $P < 0.001$ vs. HG cells without inhibitors. **D:** Supershift and competition assays. EMSA was performed as described in the text. Lane 1 shows nuclear extracts (NE) of NG-treated cells, subsequent to stimulation with HG for 3 h (lane 2), pretreated with 10 $\mu\text{mol/l}$ sorbinil or tolrestat followed by stimulation with HG for 3 h (lanes 3 and 4), NE of HG treatment + PBS (lane 5), and supershift with p65 antibody or the preimmune sera (PIS) (lanes 6 and 7). Lanes 8 and 9 show competition with $\times 20$ and $\times 50$ unlabeled oligonucleotide probe; lane 10 shows NE alone without oligonucleotide probe.

shifted the band to a higher molecular weight. The preimmune serum had no effect on NF- κB mobility (Fig. 1D). In addition, excess (20- and 50-fold) cold NF- κB oligonucleotide completely eliminated both the p50 and p65 bands, indicating that these were specifically due to NF- κB (Fig. 1). These observations validate the fact that the specific activity reported by EMSA is entirely due to NF- κB activation. As shown in Fig. 1A, $\sim 65\%$ of the high-glucose-induced NF- κB activation was prevented by 10 $\mu\text{mol/l}$ sorbinil, suggesting that inhibition of aldose reductase prevents high-glucose-induced NF- κB activation.

For determining the minimum duration of sorbinil exposure required to prevent the effects of high glucose,

VSMCs were incubated with 10 $\mu\text{mol/l}$ sorbinil for 0–48 h before stimulation with high glucose for 3 h. Significant inhibition of high-glucose-induced NF- κB activation in cells that were preincubated with sorbinil for 12 h was observed. However, for maximal inhibition, 24-h pretreatment was necessary (Fig. 1B). No significant inhibition of NF- κB was observed when the cells were treated with sorbinil and high glucose at the same time (Fig. 1C). These results demonstrate that NF- κB inhibition by sorbinil is independent of the extent to which the pathway is activated and that the inhibition requires prolonged preincubation, suggesting that changes in metabolism and/or gene expression may be necessary for sorbinil to prevent the

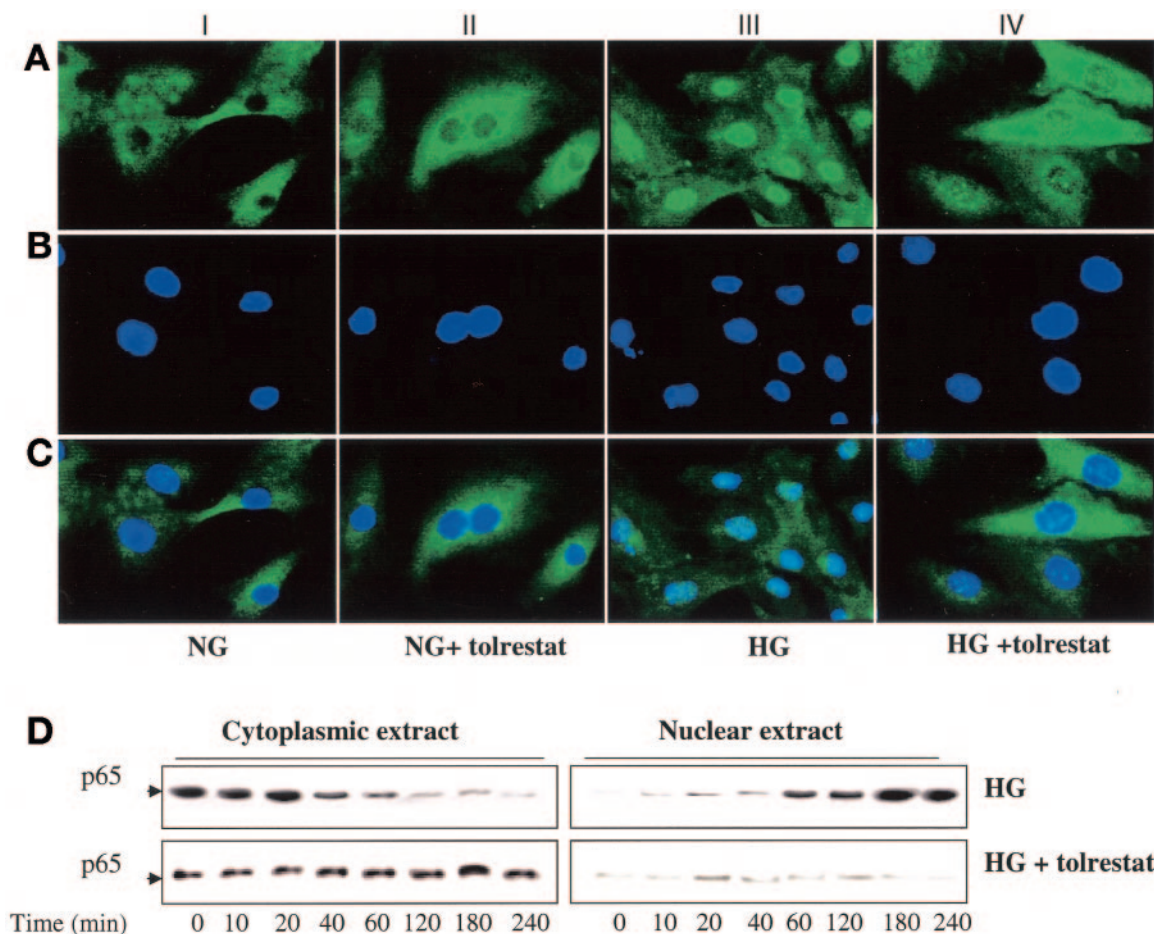


FIG. 2. Inhibition of aldose reductase prevents the high glucose (HG)-induced nuclear translocation of NF- κ B. The growth-arrested VSMCs in normal glucose (NG) were left untreated (*I*), pretreated with tolrestat for 24 h (*II*), treated with an additional 19.5 mmol/l glucose for 3 h (*III*), or preincubated with 10 μ mol/l tolrestat for 24 h followed by stimulation with HG for 3 h (*IV*). Three of such independent experimental sets were performed, and one of the representative photomicrographs ($\times 60$) is shown. *A*: NF- κ B localization by immunostaining using antibodies against p65. *B*: VSMC nuclei stained with Hoechst-33342. *C*: Composite images generated by superimposing photomicrographs shown in *A* and *B*. *D*: Time course analysis of NF- κ B translocation. Western blots were developed in cytosolic and nuclear extracts prepared from serum-starved cells that were pretreated without (*top*) or with (*bottom*) 10 μ mol/l tolrestat for 24 h followed by stimulation with high glucose for the indicated times using anti-p65 antibodies.

effects of hyperglycemia. Although, culturing VSMCs in medium that contained 19.5 mmol/l mannitol also activated NF- κ B (Fig. 1C), this was not inhibited by aldose reductase inhibitors (Fig. 1C), indicating that inhibition of aldose reductase does not interrupt hyperosmotic signaling leading to NF- κ B activation.

Inhibition of aldose reductase prevents high-glucose-induced NF- κ B nuclear translocation. The signaling events that lead to NF- κ B activation involve phosphorylation, ubiquitination, and proteolytic degradation of I κ B- α from its inactive trimeric complex of I κ B/p65/p50 in the cytosol followed by translocation of the active dimer (p65/p50) to the nucleus, where it binds to cognate DNA sequences to activate transcription (23). For determining whether all or any of these events are affected by inhibiting aldose reductase, translocation of NF- κ B to the nucleus was determined by two different methods: 1) immunofluorescence staining using p65 antibodies and 2) Western blot analysis of cytosolic and nuclear extracts using antibodies against p65. As shown in Fig. 2A, most of the inactive form of NF- κ B was present in the cytosol of the VSMCs that were treated with either the vehicle or tolrestat alone. Incubation with high glucose, however, led

to nuclear localization of fluorescence, which corresponded to the intracellular staining of the Hoechst nuclear dye (Fig. 2B). This is clearly visualized in the superimposed image showing cyan nuclei arising from superimposed blue and green colors (Fig. 2C). However, when the tolrestat-pretreated cells were stimulated with high glucose, very little nuclear staining was observed and the cells continued to show diffused perinuclear staining. These results indicate that inhibition of aldose reductase prevents high-glucose-induced nuclear translocation of p65. This was further confirmed by Western analysis using p65 antibodies in the cytosolic and nuclear extract stimulation with high-glucose in the absence and presence of aldose reductase inhibitor (Fig. 2D). Within 60 min, exposure of VSMCs to high glucose alone resulted in the translocation of a significant amount of NF- κ B from cytosol to the nucleus, which was maximal at 180 min. However, in the presence of aldose reductase inhibitor, little or no translocation of NF- κ B from cytosol to nucleus was observed (Fig. 2D). Together, these data suggest that inhibition of aldose reductase prevents high glucose-induced NF- κ B activation in cultured VSMCs by abrogating signaling mechanisms that activate NF- κ B.

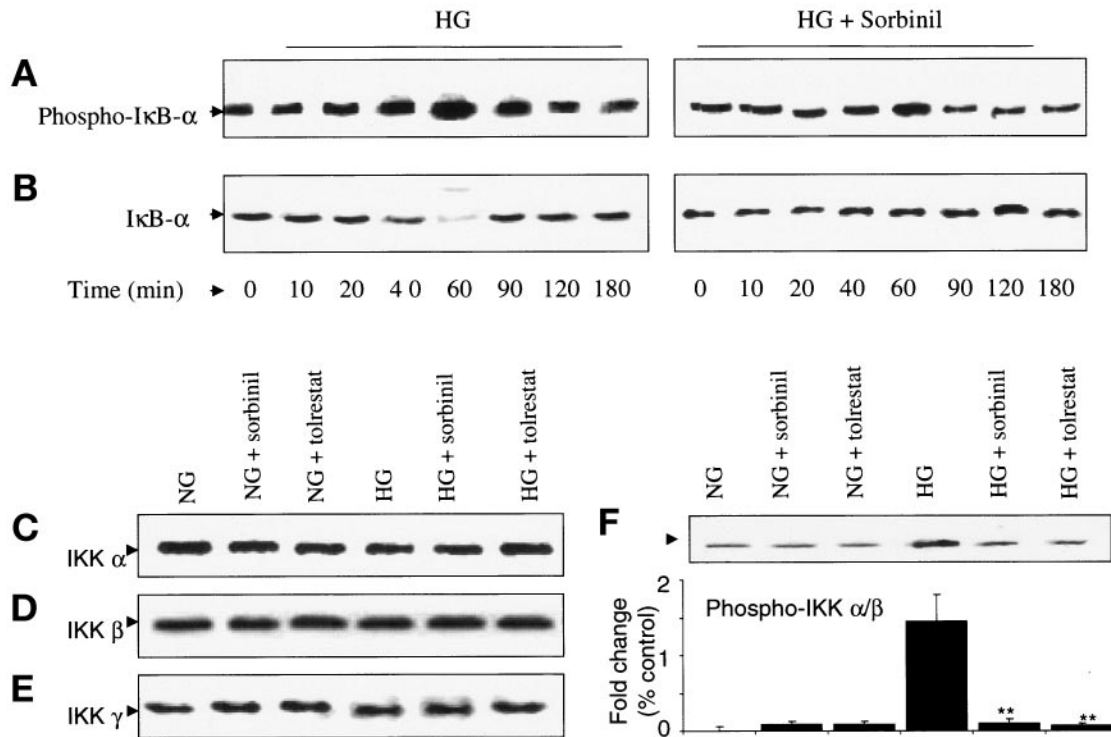


FIG. 3. Inhibition of aldose reductase prevents the phosphorylation and degradation of IκB-α and phosphorylation of IKK-α/β. The growth-arrested VSMCs without or with preincubation with aldose reductase inhibitors were treated with high glucose (HG) for the indicated time periods for measuring IκB-α phosphorylation and degradation and for 3 h for measuring IKK. The cell extracts were prepared, and equal amounts of protein obtained from a pool of three independent incubations were subjected to SDS-PAGE. Western blot analysis was performed at least three times using phospho-IκB-α (A), IκB-α (B), IKK-α (C), IKK-β (D), IKK-γ (E), and phospho-IKK-α/β (F) antibodies. NG, normal glucose. Bars represent means ± SE ($n = 3$); ** $P < 0.001$ vs. HG cells.

Inhibition of aldose reductase prevents high-glucose-induced IκB-α phosphorylation and degradation.

To elucidate the mechanism of inhibition of NF-κB signaling further, we examined signaling events upstream to nuclear translocation of activated NF-κB. For this, we determined whether the inhibition of aldose reductase prevents the phosphorylation and degradation of IκB-α. Upon stimulation of VSMCs with high glucose, partial IκB-α phosphorylation in the VSMCs was observed within 40 min, and complete phosphorylation occurred by 60 min (Fig. 3A). However, when sorbinil-pretreated VSMCs were stimulated with high glucose, little or no phosphorylation of IκB-α was observed for 180 min (maximal observation time). Because the phosphorylated IκB-α undergoes proteolytic degradation, we next determined the effect of sorbinil on the degradation of IκB-α. Upon stimulation with glucose, nearly complete degradation of IκB-α was observed in 60 min, and full resynthesis was achieved in 120 min (Fig. 3B, left). However, in sorbinil-pretreated cells, no degradation of IκB-α was observed for a total observation period of 180 min (Fig. 3B). From these data, we infer that inhibition of aldose reductase prevents high-glucose-induced phosphorylation of IκB-α and its proteolytic degradation and the translocation of the active p65/p50 (NF-κB) dimer from the cytosol to the nucleus.

Aldose reductase inhibition prevents high-glucose-induced phosphorylation of IKK-α/β. Because IκB-α is phosphorylated by IKK (23), we next asked whether inhibition of aldose reductase prevents high-glucose-induced IKK activation. To address this question, we deter-

mined high-glucose-induced changes in IKK and phospho-IKK in the absence and presence of aldose reductase inhibitors. Neither high glucose nor aldose reductase inhibitors affected the basal abundance of IKK-α, -β, and -γ proteins (Fig. 3C-E). However, incubation with high glucose increased IKK-α/β phosphorylation (as determined by Western blot analysis using anti-phospho-IKK-α/β antibody), and this phosphorylation was prevented in VSMCs that were pretreated with sorbinil or tolrestat (Fig. 3F). These results suggest that the high-glucose-induced IKK phosphorylation and activation extinguishes signaling events that lead to NF-κB activation. To determine the functional consequences of interrupting NF-κB signaling, we examined whether the end effectors of NF-κB signaling (i.e., proteins encoded by NF-κB-dependent genes) are also prevented by inhibiting aldose reductase.

Inhibition of aldose reductase prevents the induction of adhesion molecules and aldose reductase by high glucose.

Previous studies showed that NF-κB regulates the expression of adhesion molecules (26) and that diabetic vessels display increased leukocyte adherence (4,27,28) in part as a result of hyperglycemia or AGE-induced upregulation of adhesion molecules (28-30). The increase in the expression of adhesion molecules during hyperglycemia (28,31-33) seems to be mediated by NF-κB, which also could regulate aldose reductase expression (34). Hence, we examined the effects of aldose reductase inhibition on the expression of intracellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), and aldose reductase. Results shown in Fig. 4

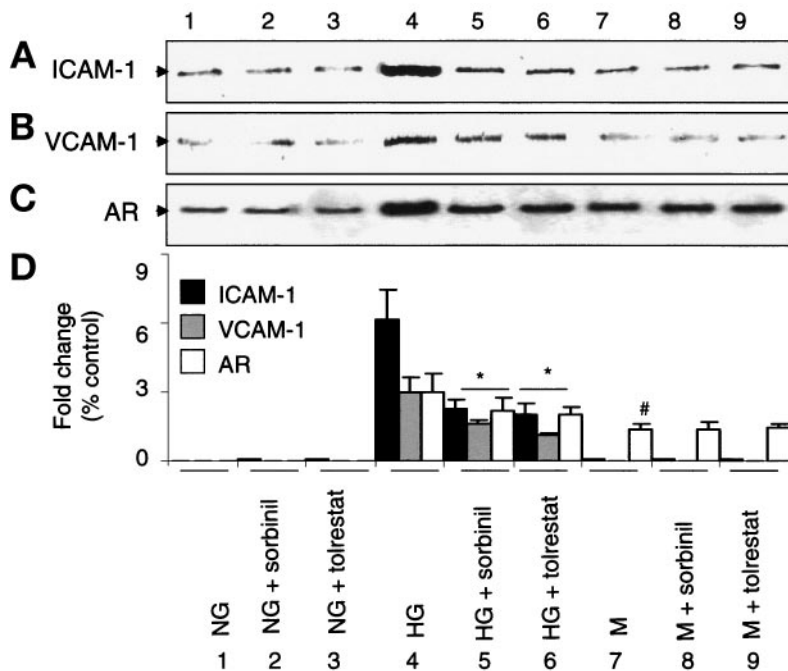


FIG. 4. Inhibition of aldose reductase (AR) prevents high glucose (HG)-induced activation of adhesion molecules. Growth-arrested VSMCs in normal glucose (NG) or HG or mannitol (M) were cultured in the absence or the presence of the aldose reductase inhibitors sorbinil or tolrestat (10 $\mu\text{mol/l}$ each) for 24 h. Pooled total cell extracts were prepared from three separate incubations; the proteins were separated by SDS-PAGE and then subjected to Western blot analysis. Western blot analysis was carried out at least three times using either monoclonal antibodies raised against ICAM-1 and VCAM-1 or polyclonal antibodies raised against human aldose reductase. The blots were visualized using enhanced chemiluminescence. Bars represent means \pm SE ($n = 3$); * $P < 0.001$ vs. HG cells.

demonstrate that compared with cells that are grown in normal glucose, cells that are cultured in the presence of high glucose express higher levels of ICAM-1, VCAM-1, and aldose reductase. However, preincubation with the aldose reductase inhibitors sorbinil and tolrestat significantly prevented high glucose-mediated induction of ICAM-1, VCAM-1, and aldose reductase. Treatment with iso-osmolar mannitol did not increase ICAM-1 or VCAM-1, suggesting that the induction of these proteins by glucose is not an osmotic effect. Mannitol treatment, however, did increase aldose reductase. The promoter of the aldose reductase gene has an osmotic response element, and the gene is sensitive to osmolarity (34–36). However, induction of aldose reductase by mannitol was not prevented by sorbinil or tolrestat, indicating that the changes in aldose reductase expression in the presence of high-glucose are not due to an increased osmolarity of the medium and are unlikely to be mediated by the activation of osmotic response element. Thus, inhibition of high-glucose-induced NF- κ B signaling by aldose reductase inhibitors also prevents the induction of key NF- κ B target genes.

Ablation of aldose reductase by siRNA. Although sorbinil and tolrestat are relatively specific aldose reductase inhibitors, their nonspecific effects cannot be excluded. We therefore examined the effects of ablating the aldose reductase by using siRNA. We designed two specific target sequences of aldose reductase (Table 1). As shown in Fig. 5, transfection of VSMCs with aldose reductase siRNA1 ablated almost 100% of aldose reductase protein expression and activity after 48 h, whereas aldose reductase siRNA2 diminished aldose reductase protein expression and activity by >95% of their initial values. After 72 h, transfection with siRNA1 or siRNA2 decreased aldose reductase expression and activity to undetectable levels. For all subsequent experiments, treatment with aldose reductase siRNA1 for 48 h was used.

Inhibition of aldose reductase prevents the high-glucose-induced activation of NF- κ B and AP-1 but

not SP-1 or OCT-1. We next determined whether siRNA silencing of aldose reductase would prevent NF- κ B activation and whether other transcription factors such as AP-1, SP-1, and OCT-1 were affected as well. As shown in Fig. 6, treatment with aldose reductase siRNA prevented NF- κ B and AP-1 activation induced by high glucose but not by mannitol. Nevertheless, the activities of SP-1 and OCT-1 were unaffected. These results suggest that inhibition of aldose reductase prevents specific signaling pathways related to NF- κ B and AP-1 activation and does not lead to a nonspecific inhibition of other transcription factors.

PKC inhibitors prevent high-glucose-induced NF- κ B activation. Activation of NF- κ B upon stimulation of cytokine receptors is mediated by multiple signaling pathways; however, mediators of glucose-induced NF- κ B activation remain unknown. We therefore tested whether high glucose would activate PKC. As shown in Fig. 7, stimulation of VSMCs with high glucose led to a progressive increase in the total PKC activity in the cells. Significant PKC activation was observed within 30 min of exposure to high glucose, and maximal activation was observed within 120 min. However, when the cells were pretreated with sorbinil, high-glucose-induced activation of PKC was completely prevented, suggesting that aldose reductase is required for high-glucose-induced PKC activation. To determine whether PKC is essential for NF- κ B activation, we treated VSMCs with the PKC inhibitors chelerythrine and calphostin C. Although PKC inhibitors did not affect basal NF- κ B activity, both chelerythrine and calphostin C prevented high-glucose-induced NF- κ B activation (Fig. 8), suggesting that high-glucose-induced NF- κ B is PKC-dependent. Significantly, mannitol-induced NF- κ B activation was not affected by PKC inhibitors, suggesting that osmotic stress activates NF- κ B by a PKC-independent pathway. Moreover, the differential sensitivity of NF- κ B activation by glucose and mannitol to PKC inhibitors indicates that glucose-induced NF- κ B activation is not due to osmotic stress.

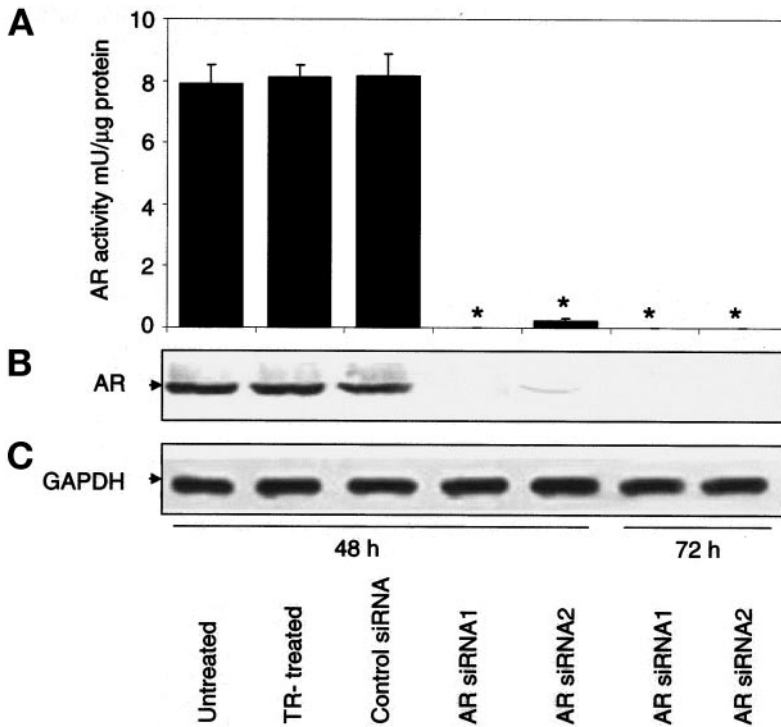


FIG. 5. Ablation of aldose reductase (AR) by siRNA. The serum-starved VSMCs were transfected with two different double-stranded aldose reductase-specific siRNAs to a final concentration of 100 nmol each and cultured for 48 h at 37°C. The VSMCs that were incubated with the transfection reagent (TR) only or with nonspecific RNA (control siRNA) were used as control. **A:** Aldose reductase activity determined using DL-glyceraldehyde and NADPH as substrates. Bars represent means \pm SE ($n = 4$); $*P < 0.001$ vs. control siRNA transfected cells. **B** and **C:** Western blots after SDS-PAGE separation of 10 μ g of VSMC cell protein developed using anti-aldose reductase or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies, respectively.

Inhibition of aldose reductase prevents the activation of NF- κ B in balloon-injured arteries of diabetic rats.

To examine changes in NF- κ B in situ, we injured carotid arteries of rats. A total of 20 diabetic and 20 nondiabetic rats were used for the study. One rat in the nondiabetic group and three rats in the diabetic group died, and their tissues were not included in the study. All other rats completed the protocol successfully. Ten and 21 days after injury, the carotid arteries were harvested, fixed, and stained with antibodies raised against activated NF- κ B. Lesions of balloon-injured arteries were associated with increased NF- κ B activity (Fig. 9). Most intense staining was associated with the luminal edge of the lesion, which corresponds to the cell population showing the highest proliferative activity. Appreciable levels of staining were observed at 10 as well as 21 days after injury, although intensity of staining seems to diminish from 10 to 21 days. Significantly higher levels of staining were associated with diabetic as compared with nondiabetic vessels

(Fig. 9). The staining in the diabetic vessels was most intense near the lumen, but cells much deeper in the intima were also stained with higher frequency. In contrast, most of the cells stained in the nondiabetic vessels were located near the lumen, and there was a progressive decline in staining from the lumen to the media. These data indicate that injured diabetic arteries display more pronounced NF- κ B activation than nondiabetic vessels.

To examine the role of aldose reductase in the activation of NF- κ B, we fed diabetic rats 15 mg \cdot kg $^{-1}$ \cdot day $^{-1}$ tolrestat. Previous studies have shown that this dose range of tolrestat ameliorates some of the diabetes complications in streptozotocin-induced diabetic rats (37,38). In the present study at 10 and 21 days after injury, the neointima-media ratios were 0.79 ± 0.07 and 1.07 ± 0.1 in nondiabetic rats and 1.04 ± 0.09 and 1.54 ± 0.13 , respectively, in diabetic rats, showing that diabetic vessels have significantly ($P < 0.05$) higher (by 30–45%) neointima formation than nondiabetic arteries. At both of the time

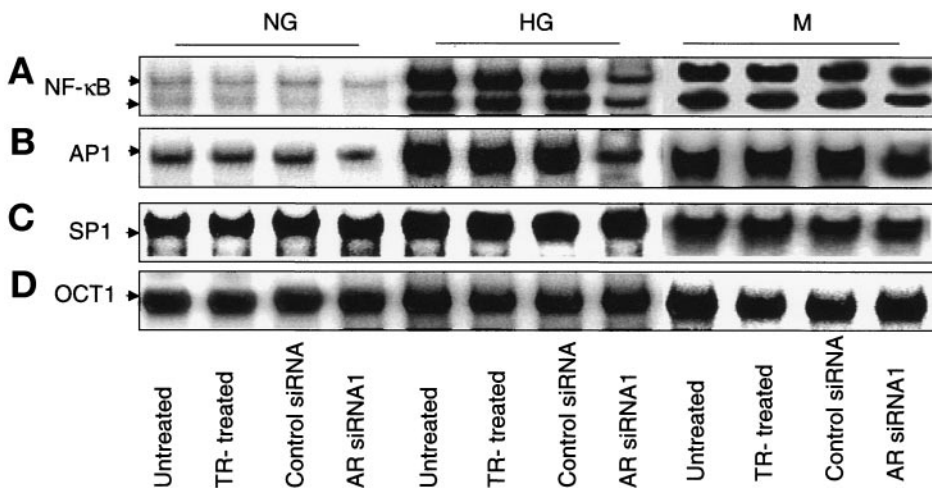


FIG. 6. Silencing of aldose reductase (AR) mRNA by siRNA transfection attenuates high glucose (HG)-induced NF- κ B and AP-1 activation but does not affect SP-1 or OCT-1 activity. Expression of aldose reductase was silenced by incubating the cells with aldose reductase siRNA1 for 48 h at 37°C. Equal amounts of nuclear extracts, prepared from the aldose reductase-ablated VSMCs in normal glucose (NG) or stimulated with HG or mannitol (M) for 3 h, were subjected to EMSA using NF- κ B (**A**), AP-1 (**B**), SP-1 (**C**), and OCT-1 (**D**) consensus sequences. Extract prepared from cells that were left untreated or treated with the transfection reagent (TR) or control siRNA were used as controls.

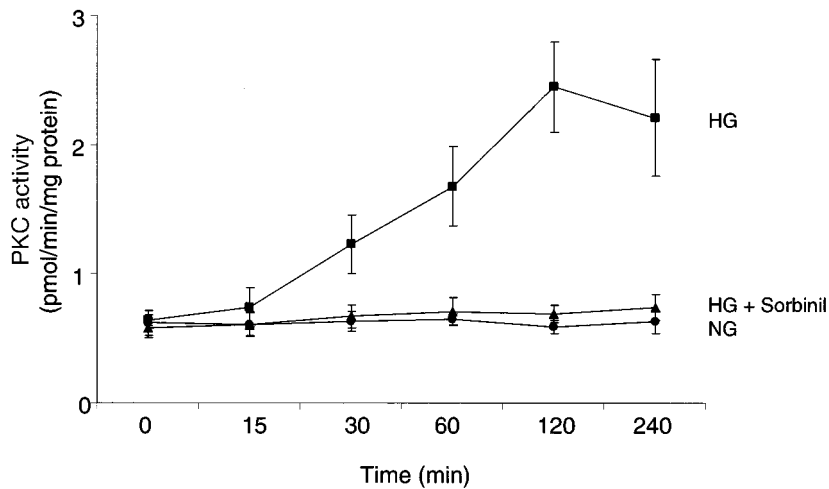


FIG. 7. Inhibition of aldose reductase prevents high-glucose-induced PKC activation. **A:** Serum-starved VSMCs were preincubated without or with 10 $\mu\text{mol/l}$ sorbinil for 24 h and stimulated with high glucose (HG) for the indicated time periods. After incubation, the membrane fraction from each sample was isolated and the PKC activity associated with each of these fractions was measured as described in the text using Promega SignaTect total PKC assay system. NG, normal glucose. Each data point represents mean \pm SE ($n = 4$).

points examined, the neointima-to-media ratio was significantly ($P < 0.05$) lower in the tolrestat-treated nondiabetic (0.55 ± 0.09 and 0.75 ± 0.07 , respectively) and diabetic (0.77 ± 0.09 and 1.21 ± 0.11) rats than the untreated rats. The observation that inhibition of aldose reductase diminishes neointima-to-media ratio in balloon-injured arteries of nondiabetic rats is consistent with our previous report showing that sorbinil treatment prevents neointimal hyperproliferation in the same model. In addition, our present report shows that inhibition of aldose reductase decreases neointima formation in diabetic vessels as well. Tissue sections obtained from tolrestat-treated rats displayed a substantial decrease in the staining with the anti-activated NF- κ B antibody. In sections that were obtained both 10 and 21 days after injury, tolrestat significantly inhibited NF- κ B staining (Fig. 9). Treatment with tolrestat also prevented staining of the inner uninjured portions of the artery. There was no statistically significant difference between the extent of staining in control and diabetic vessels after tolrestat treatment. These data suggest that diabetes enhances and accelerates the inflammatory response to arterial injury

and that inhibition of aldose reductase prevents the hyperactivation of NF- κ B caused by diabetes.

DISCUSSION

Previous studies have led to the identification of multiple hyperglycemia-induced changes in metabolism and signaling that have been linked to increased formation of reactive oxygen species and AGEs and activation of aldose reductase and PKC (1–3). These changes are associated with the increased activity of transcription factors such as NF- κ B and AP-1 and synthesis of cytokines, adhesion molecules, and growth factors (2,3). Nevertheless, it remains unclear whether the changes in signaling and metabolism are unrelated and independent manifestations of hyperglycemic injury or are interlinked or sequential. This distinction, however, is imperative for understanding and treating secondary diabetes complications. In this regard, the present study provides clear evidence supporting the notion that hyperglycemia-induced activation of the polyol pathway; PKC, NF- κ B, and AP-1; and increased expression of adhesion molecules are linked events that are sequen-

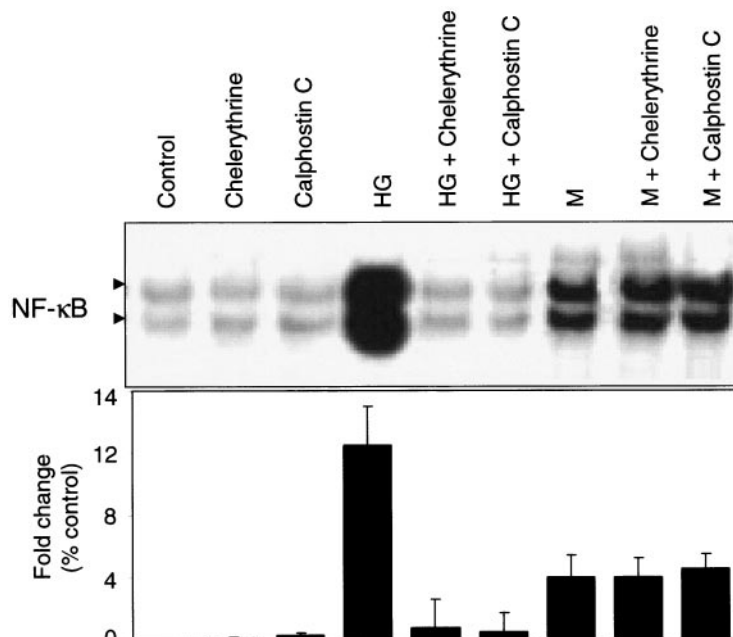


FIG. 8. Inhibition of PKC attenuates high glucose (HG)-induced NF- κ B activation. Serum-starved VSMCs were preincubated without or with the PKC inhibitors chelerythrine chloride (2 $\mu\text{mol/l}$) and calphostin C (0.2 $\mu\text{mol/l}$), respectively, for 1 h followed by incubation with HG (25 mmol/l) or mannitol (M) (19.5 mmol/l) for an additional 3 h. Equal amounts of nuclear protein obtained from a pool of three independent incubations were subjected to EMSA as described in RESEARCH DESIGN AND METHODS. Data represent means \pm SE of three independent analyses; * $P < 0.001$ vs. cells that were incubated with HG in the absence of PKC inhibitors.

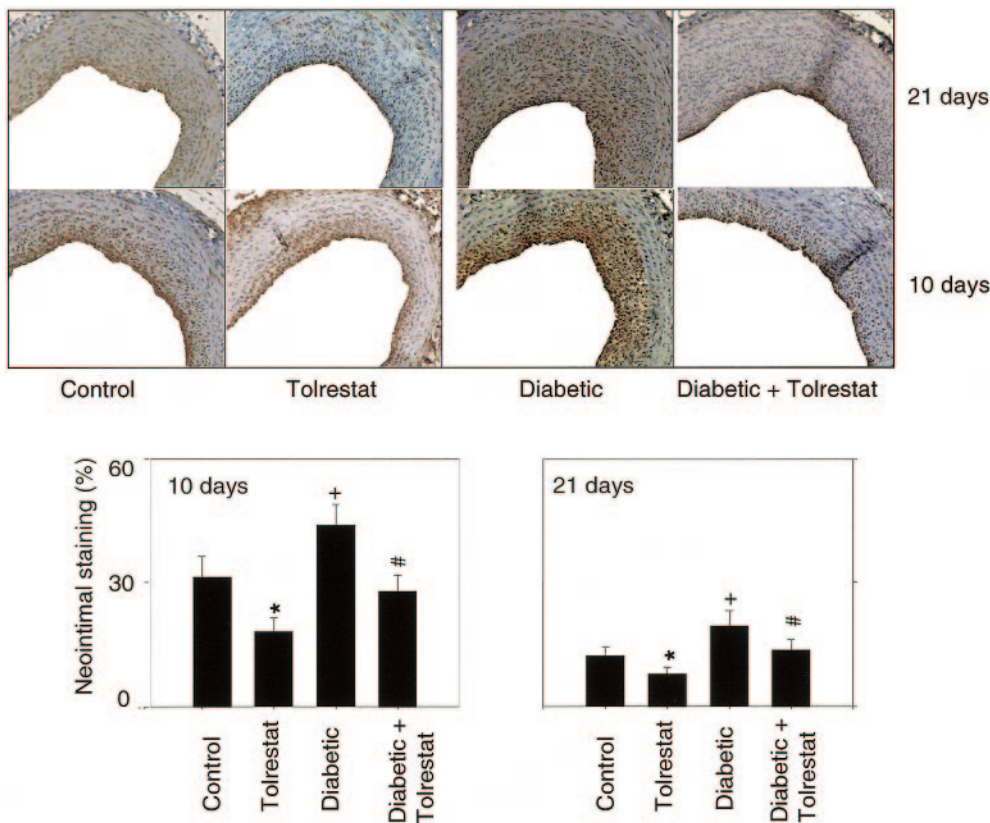


FIG. 9. Inhibition of aldose reductase prevents NF- κ B activation in balloon-injured rat arteries. Cross-sections of balloon-injured rat arteries were obtained from nondiabetic and diabetic rats 10 and 21 days after balloon injury and were stained with anti-p65 antibodies. Immunoreactivity is evident as a dark brown stain, whereas the nonreactive areas display only the background color. The bar graph shows mean \pm SE values of the percentage of neointima stained by the anti-p65 antibody; * $P < 0.05$ in tolrestat vs. control; $^+P < 0.05$ in diabetic vs. control; # $P < 0.05$ diabetic + tolrestat vs. diabetic without tolrestat.

tially activated by hyperglycemia. In addition, our data suggest that activation of the polyol pathway is antecedent to the increases in PKC, NF- κ B, and AP-1 and that inhibition of this pathway extinguishes key signaling events that lead to increased vascular inflammation and adhesion.

Our data show that inhibition of aldose reductase prevents NF- κ B activation both in cells that are exposed to high glucose in culture and in situ after balloon injury. Pretreatment of VSMCs in culture with aldose reductase inhibitors decreased high-glucose-induced NF- κ B activation, which was accompanied by decreased translocation of p65 to the nucleus (Fig. 2). The inhibition of NF- κ B translocation was associated with a decrease in high-glucose-induced phosphorylation and proteolytic degradation of I κ B- α and a decrease in IKK- α/β phosphorylation (Fig. 3), indicating that inhibition of aldose reductase abrogates I κ B- α phosphorylation and thus prevents dissociation of the I κ B-p55-p65 complex but does not directly interfere with NF- κ B-dependent gene transcription. These results are consistent with our previous observations that inhibition of aldose reductase prevents tumor necrosis factor- α -induced NF- κ B activation in VSMCs (21) and suggest that inhibition of NF- κ B and its downstream effectors may be a significant aspect of the salutary effects of aldose reductase inhibitors against hyperglycemic injury and dysfunction.

To examine the downstream effects of NF- κ B inhibition, we determined whether treatment with aldose reductase inhibitors prevents the transcription and upregulation of NF- κ B-dependent genes. Although NF- κ B regulates the expression of multiple genes (26), increased transcription of genes encoding for adhesion molecules such as ICAM-1 and VCAM-1 are considered prototypical NF- κ B responses

(26), and increased expression of adhesion molecules during hyperglycemia and diabetes has been shown to be NF- κ B dependent (28,33). In agreement with previous reports on other cell types (28–33), we found that exposure to high glucose upregulates the expression of ICAM-1 and VCAM-1 in VSMCs. That this was prevented by inhibiting aldose reductase is consistent with the previous observation that treatment with aldose reductase inhibitor, which prevented glomerular hyperfiltration in diabetic rats, decreased ICAM-1 expression and mononuclear cell infiltration (38). Thus, a decrease in the expression of adhesion molecules in vivo during diabetes (39) and in cells that are exposed to high glucose in culture (this study) indicates that inhibition of aldose reductase extinguishes NF- κ B signaling.

In addition to inhibiting NF- κ B, treatment with aldose reductase siRNA prevented the high-glucose-induced activation of the transcription factor AP-1. These responses, however, do not seem to be nonspecific, because the activities of other transcription factors such as OCT-1 and SP-1 were not affected by ablating aldose reductase expression by siRNA, suggesting that aldose reductase is essential for NF- κ B and AP-1 activation by high glucose and that the effects observed are not due to the nonspecific effects of the drugs used to inhibit aldose reductase.

In agreement with previous observations, we found that high glucose increased PKC activity (1,4,5), and inhibition of PKC prevented high-glucose-induced NF- κ B activation (40,41), consistent with the view that high glucose activates NF- κ B by a PKC-dependent pathway. Significantly, high-glucose-induced PKC activation was prevented in sorbinil-pretreated cells, suggesting that aldose reductase regulates the activation of PKC by high glucose. Because

inhibition of PKC by chelerythrine abrogates high glucose-induced AP-1 activation (7), prevention of AP-1 activation in aldose reductase siRNA-treated cells may also be due to lack of PKC activation. Although the relationship between aldose reductase and PKC remains unclear, inhibition of aldose reductase by tolrestat has been shown to prevent the synthesis of DAG (42), which is an essential endogenous activator of PKC. It has been suggested that the increase in the polyol pathway, as a result of high glucose, induces a pseudohypoxic state by increasing the NADH-to-NAD⁺ ratio (1–3,5), which stimulates the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate. Because glycerol-3-phosphate is a DAG precursor, increased synthesis of glycerol-3-phosphate upon exposure to high glucose stimulates DAG synthesis (43). Alternatively, aldose reductase-dependent reduction of glyceraldehyde to glycerol may be essential for DAG synthesis. Extensive future experiments will be required to understand the role of aldose reductase in high-glucose-induced PKC activation. Nonetheless, our current observation that inhibition of aldose reductase prevents PKC activation suggests that aldose reductase is upstream of PKC activation and that inhibition of aldose reductase during diabetes could provide salutary effects similar to or greater than those provided by PKC inhibitors (1–3,5).

In addition to metabolic changes, changes in medium osmolarity could lead to NF- κ B activation (41,44). Indeed, we find that NF- κ B is activated by high glucose as well as by mannitol. However, the pathways of NF- κ B activation by the two stimuli seem to be different. Our results show that the pathways activated by high glucose are inhibited by PKC inhibitors, whereas mannitol-induced NF- κ B activation was insensitive to PKC inhibition. That glucose and mannitol stimulate divergent mediators is further supported by our observations that inhibition of aldose reductase prevents high glucose but not mannitol-induced NF- κ B activation. Moreover, in contrast to high glucose, mannitol did not enhance the expression of the adhesion molecules ICAM-1 and VCAM-1. Collectively, these observations indicate that aldose reductase activity is essential for the activation of PKC and NF- κ B, leading to increased expression of adhesion molecule, and that even though the enzyme is induced by osmotic stress, it is not essential for the osmotic stimulation of NF- κ B.

In summary, the present study demonstrates that aldose reductase is an obligatory mediator of the inflammatory changes caused by hyperglycemia and diabetes. The activity of aldose reductase seems to be an essential prerequisite for the activation of NF- κ B by high glucose and that aldose reductase is a critical determinant of inflammatory responses of injured diabetic vessels. Although the processes by which aldose reductase influences NF- κ B activation remain unclear, our results demonstrate that inhibition of aldose reductase extinguishes signaling events that lead to PKC and NF- κ B activation and those that enhance the expression of adhesion molecules. Thus, aldose reductase inhibition may represent a novel therapeutic modality for the treatment of diabetic vascular complications such as atherosclerosis and restenosis.

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