High-glucose-induced nuclear factor κB activation in vascular smooth muscle cells

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Received 6 October 1999; accepted 7 December 1999

Abstract

Objective: Vascular smooth muscle cell (VSMC) dysfunction plays a role in diabetic macrovasculopathy. This dysfunction may be caused or exacerbated by expression of many of genes potently activated by the transcriptional factor nuclear factor κB (NF-κB). We have examined whether culture of VSMCs under high glucose conditions to stimulate the diabetic state can lead to the activation of NF-κB.

Methods: NF-κB activation was assessed in VSMCs stably transfected with a cis-reporter plasmid containing the NF-κB binding sites.

Results: Within 3-h incubation, high glucose (27.5 or 55 mmol/l) alone induced an increase in NF-κB activity in VSMCs; this increase was mimicked by mannitol given to deliver the same osmolar stress to the cells. High glucose or mannitol also enhanced TNFα-stimulated NF-κB activity. Incubation with high glucose for 48 h followed by stimulation with TNFα led to a marked potentiation of NF-κB activation compared with normoglycemic (5.5 mmol/l) VSMCs exposed to TNFα, while mannitol attenuated this effect. A 48-h incubation with high glucose substantially reduced glutathione (GSH) levels compared with normoglycemic VSMCs, whereas mannitol significantly increased GSH levels. An antioxidant N-acetyl-L-cysteine and a selective protein kinase C (PKC) inhibitor GF109203X significantly suppressed the TNFα-induced NF-κB activation, and abrogated potentiation of TNFα-induced NF-κB activity caused by high glucose (27.5 mmol/l). Conclusion: These results suggest that acutely high glucose causes alterations in osmolarity leading to activation of NF-κB, but that exposure to high glucose for more prolonged times causes changes in antioxidant defences and activation of PKC, which potentiates cytokine activation of NF-κB. Further definition of these pathways will help to delineate important signals mediating the aberrant behavior of VSMCs under hyperglycemic/diabetic conditions. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Vascular smooth muscle cells; Nuclear factor κB; High glucose; Glutathione; Protein kinase C

1. Introduction

Cardiovascular disease is the leading cause of mortality in patients with diabetes [1]. Myocardial infarction and stroke are the primary cause of death in as many as 80% of patients with non-insulin-dependent diabetes mellitus [2]. The outcome of the Diabetes Control and Complications Trial [3] appears to have effectively resolved the issue of whether chronic hyperglycemia is an important factor in microvasculopathy. Although other studies have also demonstrated the importance of hyperglycemia in macrovascular disease, the mechanisms through which hyperglycemia affects diabetic macroangiopathy have yet to be identified.

The potential importance of the nuclear factor κB (NF-κB) system as a key player in control of transcription of genes encoding mediators of a variety of inflammatory responses, including those leading to atherogenesis and thrombogenesis, has been a topic of broad interest. The proteins of the NF-κB family form the inactive heterodimeric complexes in the cytoplasm of cells. Activation of NF-κB results from the dissociation of these inactive complexes from their inhibitory proteins and translocation of the active inhibited NF-κB complexes to the nucleus [4–6].

One of the most potent stimuli for NF-κB activation is oxidative stress [7]. The oxidative stress seen in diabetes and hyperglycemia appears to cause increased expression of various genes which are NF-κB dependent [8–11]. In this study, we sought to: (1) investigate whether high...
glucose concentrations elicit NF-κB activation in aortic vascular smooth muscle cells (VSMCs), (2) assess the effects of exposure of VSMCs to high glucose concentrations on NF-κB activation caused by cytokines and (3) identify molecular determinants and intracellular events involved in glucose-induced alteration of NF-κB activation.

2. Methods

2.1. Cell culture

VSMCs were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar rats [12]. Cultures were fed twice weekly with Dulbecco’s modified minimal essential medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 μg/ml pipercillin and 100 μg/ml streptomycin). Cells in passages 10–15 were used for experiments.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1985).

2.2. NF-κB activation

To study NF-κB activation, cells were stably transfected with a cis-reporter plasmid containing the luciferase reporter gene linked to five repeats of an NF-κB binding site (pNFκB-Luc: Stratagene, La Jolla, CA, USA). For this, the pNFκB-Luc plasmid was transfected together with a pSV2 neo helper plasmid (Clontech, Palo Alto, CA, USA) into rat VSMCs using the FuGEN 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). The cells were cultured in the presence of G418 (Clontech) at a concentration of 500 μg/ml with medium replacement at 2–3-day intervals. Approximately 3 weeks later, G418-resistant clones were isolated using a cloning cylinder and analyzed individually for expression of luciferase activity. Thus, several clones were selected for analysis of NF-κB activation. Luciferase activity was measured using a Luciferase assay kit (Stratagene).

For immunohistochemical staining, VSMCs were untreated or treated with tumor necrosis factor-α (TNFα) for 2 h, fixed with 4% neutral phosphate-buffered formaldehyde for 20 min at room temperature, and then treated with 0.2% Triton X-100 in phosphate-buffered saline for 5 min. Cells were incubated with 1% bovine serum albumin in phosphate-buffered saline for 10 min and then incubated with a rabbit polyclonal antibody specific for the NF-κB p50 subunit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min. Cells were then incubated with green-fluorescent Alexa 488 conjugate (goat anti-rabbit IgG, Molecular Probe, Eugene, OR, USA) for 30 min and, after further washings, analyzed under a fluorescence microscope (Olympas A×80).

Electrophoretic mobility shift assay (EMSA) was performed with VSMCs untreated or treated with TNFα for 2 h. Nuclear proteins from these cells were isolated according to the method of Schreiber et al. [13], and was subjected to EMSA using 32P-labeled NF-κB double-strand oligonucleotide. Nuclear proteins were incubated with the oligonucleotide for 30 min, subjected to polyacrylamide gel electrophoresis, and autoradiographed.

2.3. GSH measurement

The monolayers were rinsed twice with ice-cold phosphate-buffered saline, and the cells were collected using a disposable cell scraper. The cells were centrifuged at 225 g for 5 min, and cell pellets were resuspended in ice-cold metaphosphoric acid solution (5%). Sonication was performed with two 15-s bursts on a Branson sonicator. The cell homogenate was centrifuged at 3000 g, 4°C for 10 min and aliquots of the resulting supernatant were taken for GSH measurements. The GSH analysis was performed using a colorimetric assay kit for glutathione (Oxis, Suite, OR, USA). Total protein analysis was performed using the Bio-Rad dye binding assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. The results of the GSH assay were standardized against cell protein concentration.

2.4. Statistical analysis

Data are presented as mean±S.E.M. Multiple comparisons were evaluated by ANOVA followed by Fisher’s protected least significant difference test. Student’s unpaired t-test was used for comparisons between two experiments. A value of P<0.05 was considered statistically significant.

3. Results

To evaluate NF-κB activation, we used VSMCs stably transfected with a cis-reporter plasmid containing the luciferase reporter gene linked to NF-κB binding sites. The activation of NF-κB was assessed by measuring the luciferase activity in cell lysates. As shown in Fig. 1A, TNFα (0.05–20 ng/ml) dose-dependently induced NF-κB activation. EMSA, performed using NF-κB double stranded oligonucleotide as a probe, also confirmed the NF-κB activation by TNFα in those cells. As shown in Fig. 1B, TNFα (20 ng/ml) caused a distinct shifted band, whereas there was no distinct band in the control cells. The strong band elicited by TNFα was completely eliminated in the presence of a 100-fold molar excess of unlabeled probe. To ascertain the nuclear translocation of active NF-κB caused by TNFα, immunohistochemical staining
Fig. 1. NF-κB activation by TNFα. (A) VSMCs were transfected with pNFκB-Luc (a cis-reporter plasmid containing the luciferase reporter gene linked to five repeats of an NF-κB binding site). The cells were treated with TNFα (0.05–20 ng/ml). After 3 h, cells were lysed, and luciferase activities were measured. Data represent the mean ± S.E.M. of triplicate observations. (B) VSMCs were treated with or without TNFα (20 ng/ml) for 2 h. The nuclear protein (10 μg) was extracted and subjected to EMSA using an NF-κB double-stranded oligonucleotide without or with 100-fold excess unlabeled probe as a competitor. (C) VSMCs were treated with medium alone (a) or TNFα (20 ng/ml) (b) for 2 h. The cells were fixed and subjected to immunohistochemical staining for nuclear translocation of NF-κB; (c) secondary antibody alone.
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**Using an anti-p50 antibody was performed. Nonstimulated cells had a diffuse distribution of immunoreactive p50 within their cytoplasm. In contrast, exposure of the cells to TNF-α (20 ng/ml) resulted in dense accumulations of immunoreactive p50 within their nuclei (Fig. 1C).**

Incubation of VSMCs with increasing concentrations of glucose resulted in a modest but significant increase in NF-κB activity after 3 h. The NF-κB activity in cells treated with 27.5 and 55 mM glucose was 112 and 124%, respectively, of that in cells treated with 5.5 mM glucose. This increase was also seen in the osmotic control cells in which mannitol was added to keep the same osmolarity as that under conditions of high concentrations of glucose (22.0 or 49.5 mM mannitol with 5.5 mM glucose) (Fig. 2A). The effects of high concentrations of glucose and mannitol on NF-κB activity in TNFα-treated cells were also examined. TNFα potently increased NF-κB activity after 3 h. This increase was enhanced in the presence of increasing concentrations of glucose. The NF-κB activity elicited by TNFα in 27.5 mM and 55 mM glucose was 112% and 126% of that with 5.5 mM glucose, respectively. This potentiation of NF-κB with high concentrations of glucose was mimicked by mannitol added in concentrations to keep the same osmolarity (Fig. 2B).

Next, the effects of prolonged exposure to high concentrations of glucose and mannitol on NF-κB activity were also evaluated. VSMCs were incubated for 48 h with high concentrations of glucose or mannitol, and the cells were then incubated for 3 h in the absence or presence of TNFα. Incubation for 48 h with high concentrations of either glucose alone or mannitol alone did not alter NF-κB activity compared with that in the cells incubated with 5.5 mM glucose (Fig. 3). However, NF-κB activity in cells stimulated with TNFα was substantially increased in the cells that were incubated with high concentrations of glucose. The NF-κB activity elicited by TNFα in cells pretreated in 27.5 and 55 mM glucose was 146 and 137%, respectively, of that in cells with 5.5 mM glucose. By contrast, incubation with mannitol before TNFα stimulation significantly decreased NF-κB activity. The NF-κB activity elicited by TNFα in cells pretreated in 5.5 mM glucose + 22.0 mM mannitol and 5.5 mM glucose + 49.5 mM mannitol was 79 and 75%, respectively, of that with 5.5 mM glucose (Fig. 3).

**Effect of high concentrations of glucose and mannitol on GSH content in the cells was examined. When cultured with 5.5 mM glucose, GSH levels were 579±1.6 nmol/mg protein. In the cells incubated for 48 h with high glucose, GSH levels were substantially reduced to 415±8.8 nmol/mg protein with 27.5 mM glucose, and to 324±16 nmol/mg protein with 55 mM glucose. In contrast, incubation with mannitol for 48 h significantly increased GSH levels in the cells; 695±34 nmol/mg protein with 5.5 mM glucose + 22.0 mM mannitol, and 917±5.6 nmol/mg protein with 5.5 mM glucose + 49.5 mM mannitol (Fig. 4A). To study the causal relationship between NF-κB activation and GSH depletion after prolonged exposure to high glucose, we investigated whether depletion of GSH by exposure of VSMCs to l-buthionine-(S,R)-sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase: the key rate-limiting enzyme in GSH synthesis and whether N-acetyl-L-cysteine (NAC) to support intracellular glutathione synthesis and scavenge reactive oxygen intermediates blunted the effect of high glucose. Depletion of GSH by exposure of VSMCs to BSO (100 μM) significantly enhanced TNFα-stimulation of NF-κB activity.**

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**Fig. 2. Acute effects of glucose and mannitol on NF-κB-dependent transcriptional activity. (A) NF-κB activity after 3-h exposure of VSMCs to high concentrations of glucose (open circles) or mannitol (open triangles; 22.0 or 49.5 mM mannitol with 5.5 mM glucose). (B) NF-κB activity after 3-h exposure of VSMCs to TNFα together with high concentrations of glucose (closed circles) or mannitol (closed triangles; 22.0 or 49.5 mM mannitol with 5.5 mM glucose). Data represent the mean±S.E.M. of triplicate observations. *P<.05 and **P<.01 compared with control (5.5 mM glucose).**
Fig. 3. Effects of prolonged exposure to high concentrations of glucose and mannitol on NF-κB-dependent transcriptional activity. VSMCs were incubated for 48 h with high concentrations of glucose (black bars) or mannitol (white bars: 22.0 or 49.5 mM mannitol with 5.5 mM glucose), and were further incubated for 3 h in the absence and presence of TNFα to measure NF-κB activity. Data represent the mean ± S.E.M. of triplicate observations. *P < .05 and **P < .01 compared with control (5.5 mM glucose).

activity (Fig. 4B). While NAC (1 mM) markedly attenuated the TNFα-induced NF-κB activity in control cells (treated with a normal glucose concentration), it prevented the effect of high glucose, resulting in the same levels of NF-κB activity as those decreased by NAC treatment in control cells (Fig. 4C).

NF-κB activation may occur in response to protein kinase C (PKC)-mediated cellular responses, which appear to be activated under high glucose conditions. We examined the effect of the PKC activator phorbol 12-myristate 13-acetate (PMA) on TNFα-stimulation of NF-κB activity and the effect of the specific PKC inhibitor GF109203X (2 μM) on TNFα-induced NF-κB activity after a 48-h incubation of cells with high glucose. As shown in Fig. 5A, PMA clearly enhanced NF-κB activation by TNFα. GF109203X significantly depressed the TNFα-induced NF-κB activity in both control cells (treated with a normal glucose concentration) and cells treated with high glucose (27.5 mM) to similar levels (Fig. 5B).

To examine whether the hexosamine biosynthetic pathway (HBP) is involved in the alteration of NF-κB activity induced by high glucose exposure, cells with or without TNFα stimulation were incubated for 3 h in the presence of different concentrations of d-glucosamine (0.1–1 mM). d-Glucosamine had no effect on NF-κB activity in either unstimulated or stimulated cells (Fig. 6). In addition, azaserine (5 μM) (an inhibitor of glutamine:fructose-6-phosphate aminotransferase, the key enzyme in the HBP) had no effect on the NF-κB activity enhanced by high glucose exposure (data not shown), suggesting that the HBP is not required for this effect.

4. Discussion

The transcription factor NF-κB has an important function in the regulation of a variety of genes involved in the inflammatory and proliferative responses of cells. Recent studies strongly indicate that the inducible transcription factor NF-κB is involved in the pathogenesis of atherosclerosis [14–16]. Activated NF-κB is present in the fibrotic thickened intima-media and atheromatous areas of the atherosclerotic lesion, within cells including VSMCs,
Fig. 4. Effects of high concentrations of glucose and mannitol on GSH content in the cells and the effects of BSO and NAC on NF-κB-dependent transcriptional activity. (A) VSMCs were incubated for 48 h with high concentrations of glucose (27.5 or 55 mM) or mannitol (22.0 or 49.5 mM mannitol with 5.5 mM glucose), and then cells were homogenized to measure their GSH contents. The GSH results were standardized against cell protein concentration. (B) VSMCs were incubated for 24 h with (open circles) or without (closed circles) BSO (100 μM), and were then stimulated with TNFα for 3 h to measure NF-κB activity. (C) VSMCs were incubated for 48 h with normal (5.5 mM) or high (27.5 mM) concentrations of glucose, and were then stimulated with TNFα for 3 h in the absence (black bars) or presence (hatched bars) of NAC (1 mM; B) to measure NF-κB activity. Data represent the mean±S.E.M. of triplicate observations. *P<.05 and **P<.01 compared with control.

whereas little or no activated NF-κB can be detected in vessels lacking atherosclerosis. A variety of molecules have been identified in the atherosclerotic environment that are able to activate NF-κB in vitro. Furthermore, an increased expression of numerous genes known to be regulated by NF-κB has been found in the atherosclerotic lesion [14–16]. NF-κB mainly consists of a dimer of the two subunits p50 and p65 (Rel A), and in its inactive state it is sequestered in the cytoplasm with an inhibitor protein called Iκ-B. Activation of NF-κB leads to the release of the inhibitory Iκ-B subunit from the heterotrimeric complex followed by translocation of the dimer to the nucleus [4–6]. Once translocated, NF-κB transcriptionally activates genes involved in the immune and inflammatory response. We confirmed substantial activation of NF-κB in response to TNFα in VSMCs by EMSA and immunohistochemistry. We then used VSMCs that were stably transfected with an NF-κB-responsive reporter gene in order to evaluate NF-κB activity.
Fig. 5. Effect of PMA and GF109203X on NF-κB-dependent transcriptional activity. (A) VSMCs were stimulated with TNFα for 3 h in the absence (open circles) or presence (closed circles) of PMA (100 nM) to measure NF-κB activity. (B) VSMCs were incubated for 48 h with normal (5.5 mM) or high (27.5 mM) concentrations of glucose, and were then stimulated with TNFα for 3 h in the absence (black bars) or presence (hatched bars) of GF109203X (2 μM) to measure NF-κB activity. Data represent the mean±S.E.M. of triplicate observations. **P<0.01 compared with control.
κB activation quantitatively. In those cells, TNFα stimulated the reporter gene expression in a concentration-dependent manner. Here we evaluated the effects of high concentrations of glucose on NF-κB activity.

Within hours of incubation, high glucose alone induced an increase in NF-κB activity in VSMCs. This increase was mimicked by mannitol, given to deliver the same osmolarity to the cells. In addition, high glucose enhanced TNFα-stimulated NF-κB activity; this enhancement was also mimicked by mannitol. These observations suggest that the acute effect of glucose is likely to be caused by a mechanism different from that of TNFα and to be associated with changes in osmolarity. Recently, two groups reported that glucose increases the NF-κB activity in vascular endothelial cells [11,17]. These two papers showed an acute effect of high glucose (incubation for 1–2 h in 30–35 mM glucose), but it is unclear whether those results were due to a metabolic or an osmotic effect. Our data suggest that the acute effects of high glucose on VSMCs NF-κB activation are largely a consequence of increased osmolarity, although the molecular mechanism by which osmolar stress leads to NF-κB activation remains to be elucidated. By contrast, glucose and mannitol exerted differing effects on TNFα-stimulated NF-κB activity when cells were exposed for a prolonged period (48 h). Incubation with high glucose led to potentiation, while incubation with mannitol resulted in attenuation of TNFα-stimulated NF-κB activity. This effect of sustained high glucose on NF-κB activation is not associated with changes in osmolarity. Since it has been documented that elevated glucose concentrations may cause cell dysfunction through the generation of free radicals, it is also possible that in our experimental conditions oxidant stress due to hyperglycemia is responsible for the activation of NF-κB [18,19].

GSH is one of the most important intracellular antioxidants. In the presence of high glucose (27.5 and 55 mM), there was an approximate 35 and 50% reduction of GSH. The mechanism for this reduction in GSH in VSMCs remains unclear. It has been consistently reported that GSH is decreased in patients with diabetes [20,21]. Various mechanisms may be responsible for this decrease, including reduced activity of glutathione reductase secondary to polyol pathway activation leading to reduced NADPH availability, and reduced activity of the key rate-limiting enzyme in GSH synthesis, γ-glutamylcysteine synthetase (γ-GCS) [22]. In the present study, BSO enhanced TNFα stimulation of NF-κB activity, while NAC attenuated the effect of high glucose in VSMCs. The enhancement of TNFα-induced NF-κB activity caused by high glucose seen in our experiment could, therefore, be partially attributed to a reduction in GSH. Consistent with these
observations, it has been reported that the glutathione redox cycle is the key step to scavenging $H_2O_2$, a known intracellular intermediate for NF-$\kappa B$ activation [23], and GSH-dependent degradation of $H_2O_2$ is impaired in vascular endothelial cells in media containing high glucose [24]. On the other hand, mannitol may act not solely as a hyperosmolar agent, but could also be an effective scavenger of the oxygen radicals. The effectiveness of post-ischemic reperfusion with mannitol has been well documented in previous studies [25]. Indeed, it has been shown that mannitol plays a role in attenuating reperfusion injury of the gut, demonstrated by depression of tissue malondialdehyde levels and by elevation of tissue GSH levels [26]. In our study, mannitol also increased cellular GSH levels, which may have led to an attenuated NF-$\kappa B$ response to TNF$\alpha$.

Considerable evidence now exists to support the hypothesis that high glucose activates PKC in vascular tissue [27,28]. The present study showed that the PKC activator PMA enhanced NF-$\kappa B$ activation, suggesting that PKC is involved in the activation of NF-$\kappa B$ by TNF$\alpha$. The specific PKC inhibitor GF109203X blocked the potentiation of TNF$\alpha$-induced NF-$\kappa B$ activity seen under high glucose conditions. These data suggest that a class of PKC that is sensitive to GF1098203X is involved in both the TNF$\alpha$-induced NF-$\kappa B$ activation in VSMCs and the potentiation of this activation by high glucose. However, the finding that PKC isoforms activated by high glucose are different from those activated by phorbol ester [29] suggests that further investigation of a potential PKC isozyme-dependent pathway of NF-$\kappa B$ activation is needed.

Involvement of the hexosamine pathway in physiologic regulation of the insulin-responsive glucose transport system has recently been described [30]. In the presence of hyperglycermia, flux through the HBP increases. Recent evidence shows that high glucose-induced stimulation of TGF-$\beta1$ expression in mesangial cells is mediated by HBP [31]. We tested whether glucosamine, a putative activator of in vitro glucose toxicity through acceleration of the hexosamine pathway, influences NF-$\kappa B$ activity in VSMCs. Glucosamine did not activate NF-$\kappa B$ nor did it alter cytokine-induced NF-$\kappa B$ activity in VSMCs. In addition, azaserine, an inhibitor of the key enzyme (glutamine:fructose-6-phosphate aminotransferase) had no effect on the enhancing effect of high glucose in NF-$\kappa B$ activation in VSMCs. This suggests that the HBP is not involved in the glucose-induced change in NF-$\kappa B$ activation in VSMCs.

In conclusion, this work demonstrates that glucose causes significant changes in NF-$\kappa B$ activation in VSMCs. Acutely high glucose causes alterations in osmolarity, leading to activation of NF-$\kappa B$. Exposure to high glucose for more prolonged periods causes changes in antioxidant defences and activation of PKC, which potentiates cytokine activation of NF-$\kappa B$. Although our findings might be restricted to dedifferentiated subcultured VSMCs, further examination of these pathways may help to delineate key signals mediating the aberrant behavior of VSMCs under hyperglycemic-diabetic conditions and thereby lead to the potential development of novel therapeutic modalities for accelerated diabetic vascular disease.

Acknowledgements

This work was supported in part by a grant from Japan Private School Promotion Foundation. The authors are grateful to Miss Fumie Yokotsuka and Dr. Kazumi Akimoto (Dokkyo University School of Medicine) for technical assistance.

References

[15] Bourcier T, Wolff SP, Dean RT. Glucose autoxidation and protein...


