High glucose alone, as well as in combination with proinflammatory cytokines, stimulates nuclear factor kappa-B-mediated transcription in hepatocytes in vitro

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Abstract

Diabetes mellitus is frequently associated with coagulation disorders such as coronary heart disease or stroke. We aimed to clarify the molecular mechanism whereby hyperglycemia causes the procoagulant state. The HuH7 human hepatocyte cells were treated with high glucose alone, or in combination with proinflammatory cytokines, and the effects on the activity of the transcription factor NF-κB, which mediates the expression of acute phase and coagulation-related genes, were examined. The results showed that increasing the medium glucose concentration from 3 to 24 mM significantly enhanced the NF-κB-luciferase activity by ≈40% in the presence of insulin. The effect was promoter-specific, and was not mimicked by comparable hyperosmolality with L-glucose. Proinflammatory cytokines such as interleukin-1 and TNF-α also stimulated NF-κB-dependent transcription, and showed an additive effect with high glucose. Similar effects were obtained on acute phase or coagulation/fibrinolysis-related gene promoters such as fibrinogen or PAI-1, all of which are shown to have NF-κB-mediated transcription. Finally, pretreatment of the cells with an antioxidant PDTC completely abolished the effect of high glucose, and markedly attenuated that of TNF-α, suggesting the involvement of reactive oxygen species. These results suggest that 1) high glucose as well as proinflammatory cytokines have positive effects on NF-κB-mediated transcription in an additive manner, and enhance coagulation-related gene expression, and 2) the effects are mediated, at least partly, by the generation of oxidative stress, and may be responsible for the high prevalence of thrombotic disorders in the metabolic syndrome with diabetes, hyperinsulinemia, obesity and/or inflammation.

Key words

diabetes mellitus, hyperglycemia, nuclear factor-kappaB, oxidative stress, cytokine, hypercoagulation
1. Introduction

It is well known that long-term hyperglycemia causes diabetes-specific microvascular complications, i.e. retinopathy, nephropathy and neuropathy. In addition, patients with uncontrolled diabetes mellitus sometimes suffer from thrombotic disorders like coronary or cerebrovascular obstruction, which may occur with mild or even postprandial hyperglycemia (Ceriello, 2000; Bonora, 2002; Haheim, Holme, Hjermann, & Leren, 1995). Indeed, enhanced coagulation and/or impaired fibrinolysis are reported to accompany patients with hyperglycemia (Bruno et al., 1996; Festa et al., 1999), and large population studies suggest the relationship between postprandial hyperglycemia and cardiovascular risk (Hanefeld et al., 1996; Tominaga et al., 1999; The DECODE study group, 1999; Hanefeld et al., 2000). Other risk factors such as hyperinsulinemia, obesity, and high cytokine levels in the metabolic syndrome may also contribute, to some extent, to the hypercoagulability. However, the precise molecular mechanisms regarding how individual risk factors are integrated and are eventually causative to the thrombotic disorders remain unresolved.

In this study, we focused on the effect of each risk factor, especially high glucose, on the nuclear factor kappa-B (NF-κB)-dependent transcription, using the human hepatocyte cell line in vitro. NF-κB is a transcription factor which is known to play a pivotal role in mediating the gene expression of acute phase proteins such as C-reactive protein (CRP) or serum amyloid protein A (SAA), or a variety of inflammation/coagulation-related genes such as fibrinogen or plasminogen activator inhibitor-I (PAI-1) in the liver (Lavrovsky et al., 2000). Thus, we hypothesize that high glucose by itself activates the transcriptional activity of NF-κB-dependent genes in the liver, which causes the overproduction of the proinflammatory/procoagulant proteins.

2. Materials and Methods

2.1. Cell culture and transfection

HuH7, a human hepatoma cell line, or HuH7NF, a subclone of the HuH7 cell line in
which the pNF-κB-Luc reporter plasmid containing 5 tandem repeats of NF-κB binding sites (Stratagene, La Jolla, CA) was stably incorporated, were used in this study. The characteristics of the latter cell line were previously described (Iwasaki et al., 2004). Cells were maintained in a T25 culture flask with DMEM (high glucose; Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Invitrogen) under a 5% CO2-95% air atmosphere at 37°C. Culture medium was changed twice a week, and the cells were subcultured once a week. In some experiments (Figs. 4 and 7), HuH7 cells were transfected transiently with RSV-LTR-, human fibrinogen 5’-promoter (≈1 kb)-, or human plasminogen activator inhibitor-1 (PAI-1) 5’-promoter (≈0.7 kb)-luciferase fusion genes, by a lipofection method using a commercially available reagent [FuGene 6, Roche Diagnostics, Penzberg, Germany; Reagent (μl): DNA amount (μg)=2:1].

2.2. Experiments

The HuH7NF (or HuH7) cells were plated with ≈50% confluency and cultured in DMEM (high glucose) supplemented with 1% FBS in 24-well plates. After 48 h, the culture medium was changed to DMEM containing 1% FBS and 3 mM glucose, and then the cells were cultured with different concentrations of glucose (3 to 24 mM) according to the experimental protocol until the end of each experiment. The culture medium was also supplied with human insulin (1 nM; Sigma, St. Louis, MO) except one experiment (see the legend of Fig. 2). In some experiments (Figs. 6 and 8), the cells were simultaneously incubated with human interleukin-1β (IL-1β; PeproTech, Rocky Hill, NJ) or tumor necrosis factor-α (TNF-α; PeproTech) for the defined time interval.

2.3. Assays

Luciferase assay was performed as previously described (Aoki et al., 1997), and light output was measured for 20 sec at room temperature using a luminometer (Berthold Lumat LB9501, Bad Wildbad, Germany). Protein assay was performed using a commercially available kit (BCA Protein Assay Kit, Pierce, , Rockford, IL).
2.4. RT-PCR

Endogenous expression of the two major components of NF-κB (p65, p50), fibrinogen, PAI-1, and insulin receptor were examined by RT-PCR using Superscript II (Invitrogen) and Taq DNA polymerase (Takara, Kyoto, Japan). The primer sets used were as follows: sense, 5’-TCAATGGCTACAGGACCA-3’ and antisense, 5’-CAGCTCACCTGGAAGCA-3’ for p65; sense, 5’-CACCTAGCTCACAAGAAG-3’ and antisense, 5’-AGGCTCAAGTTCTCCACCA-3’ for p50; sense 5’-GACAACCTGCTGATCGTAG-3’ and antisense, 5’-TCATGTGTAAATCGATGCTC-3’ for fibrinogen; sense, 5’-CTTGCTTTGGTGAAGGCT-3’ and antisense, 5’-TGTGCTTCCAAGTCATTG-3’ for PAI-1, and sense, 5’-CCTCAAGAGATGATCAGTG-3’ and antisense, 5’-TGTTCAATTAGACAGGCTTGGT-3’ for insulin receptor.

2.5. Electromobility shift assay (EMSA)

EMSA was carried out using a commercially available non-RI EMSA kit (LightShift Chemiluminescent EMSA kit; Pierce). Briefly, cells were incubated with DMEM containing 3 mM glucose for 22 h, and then treated for 2 h with DMEM containing either 3 or 24 mM glucose for 2 h. Nuclear extract was prepared using NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL). The extract was then incubated for 6 h with the double-stranded, 3’-end-biotinylated oligonucleotide probe (50 fmol) encompassing the consensus NF-κB binding sequence (sense, 5’-AGTGGAGGGACTTCCCAGGC-3’ biotin; antisense, 5’-GCCTGGGAAGCTCCTCAACT-3’ biotin), and the mixture was subjected to 4% nondenaturing polyacrylamide gel (160V for 4 h). Finally, the biotinylated DNA was transferred to a nylon membrane, cross-linked, and then the biotin-labeled DNA was detected with digital imaging apparatus (LightCapture, ATTO, Japan).

2.6. Statistical analysis

Results are expressed as mean ± SEM of triplicate or quadruplicate dishes in each group. The significance of difference between mean values was evaluated by one-way ANOVA.
3. Results

3.1. Expression of NF-κB subunits and inflammation/coagulation-related proteins in HuH7 human hepatoma cell line

We first analyzed the presence of each component of NF-κB as well as hepatic inflammation-/coagulation-related proteins by RT-PCR. As shown in Fig. 1, we found PCR products with the appropriate band size in all cases, indicating the endogenous expression of mRNAs for the proteins examined.

3.2. High glucose concentration stimulates NF-κB-dependent transcription

We then examined the dose-response and time-course effects of the increase in the glucose concentration of the culture medium on NF-κB-dependent transcription using HuH7NF cells. As shown in Fig. 2A, the increment in the glucose concentration for 6 h significantly stimulated the NF-κB-luciferase activity in a dose-dependent manner. This was more obvious when the cells were simultaneously treated with insulin (1 nM), and an approximately 40% increase was observed with the increase in the glucose concentration from 3 to 24 mM. The effect seems not to be due to a difference in the cellular growth rate, because cellular protein level was not influenced by the glucose concentration, insulin, or IGF-I (used as a control) during the incubation period (Fig. 2B). A time-course experiment showed that a significant rise was obtained as early as 3 h, and reached the maximal effect at and after 6 h (Fig. 3). These results suggest that high glucose concentration per se is an independent stimulus for NF-κB-dependent transcription, and the effect is more obvious with the presence of insulin.

3.3. Specificity of high glucose on NF-κB-dependent transcription

To confirm the specificity of the above findings, we examined the effect of L-glucose on NF-κB-dependent transcription, and also the effect of D-glucose on RSV-LTR promoter. As
shown in Fig. 4, the effect of glucose (D-glucose) was not mimicked by equimolar concentration of metabolically inactive L-glucose. Furthermore, high concentration of glucose (D-glucose) did not influence the RSV-luciferase activity. These results suggest that the positive effect of high glucose is not a non-specific nutritional or osmogenic effect, but is rather a promoter- and glucose-specific event.

3.4. High glucose enhances DNA binding of NF-κB

To see if high glucose enhances the DNA binding of NF-κB, EMSA analysis was carried out using canonical NF-κB binding sequences as probes. As shown in Fig. 5, high glucose treatment (24 mM for 2 h) clearly enhanced the protein binding to the probe, compared with the control (3 mM for 2 h).

3.5. High glucose and proinflammatory cytokines have a combined effect on NF-κB-dependent transcription

It is well known that proinflammatory cytokines stimulate the transcription of acute phase protein and other hepatic inflammation-related proteins through NF-κB activation. We therefore studied the effect of representative cytokines such as IL-1β or TNF-α alone or in combination with high glucose. As shown in Fig. 6, both IL-1β (100 pM, 12 h) and TNF-α (100 nM, 12 h) potently stimulated NF-κB-luciferase activity. Again, high glucose (24 mM) with insulin (1 nM) alone caused a 40% increase, and additive effects were observed when high glucose and either cytokine were simultaneously used. These results suggest that a combination of the stimulants (cytokines, high glucose, high insulin) exerts cumulative effects on the NF-κB-dependent transcription.

3.6. High glucose also stimulates the transcription of the genes encoding coagulation-related proteins

The expression of fibrinogen and PAI-1, major regulatory proteins for coagulation, are enhanced by inflammatory stimuli. We thus examined the effect of high glucose on the 5′-promoter activity of the genes. As shown in Fig. 7, the transcriptional activity of both PAI-1
and fibrinogen genes was invariably stimulated by 24 mM glucose. These data suggest that high glucose concentration enhances the expression of hepatic proteins involved in coagulation/fibrinolysis. Since NF-κB is shown to play a major role in the transcriptional regulation of these genes, we assume that the positive effects are, at least partly, mediated through the activation of NF-κB.

3.7. The effect of high glucose is eliminated by an antioxidant PDTC

Finally, to see the possible involvement of free radical generation in high glucose-induced NF-κB activation, we carried out a similar experiment in the presence of an antioxidant PDTC. As shown in Fig. 8, the positive effect of high glucose was completely abolished, and that of TNF-α was markedly impaired under the treatment with 100 μM of PDTC. Since high glucose is known to cause enhanced mitochondrial oxidation with radical generation (Nishikawa et al., 2000), and NF-κB is a radical-sensitive transcriptional factor (van den Berg et al., 2001), we assume that elevated glucose concentration stimulates NF-κB-dependent transcription through increased oxidative stress. Our data also suggest that a similar mechanism is, at least partly, involved in the TNF-α-mediated NF-κB activation in hepatocytes.

4. Discussion

In this study, we showed the stimulatory effect of high glucose on NF-κB-dependent transcription in hepatic cells in vitro, suggesting that glucose by itself is responsible for the activation of inflammation/coagulation-related protein expression, at least partly via increased oxidative stress. These data are in accordance with the recent clinical notion that postprandial hyperglycemia is a possible risk factor for macrovascular disorders frequently seen in patients with mild diabetes mellitus (Hanefeld et al., 1996; Tominaga et al., 1999; The DECODE study group, 1999; Hanefeld et al., 2000). Furthermore, the effect was more pronounced in the presence of insulin, and was additive with the effects of proinflammatory cytokines which are additional risk factors associated with visceral obesity (Hotamisligil, 2000; Matsuzawa, Funahashi, & Nakamura, 1999). This may explain the molecular
mechanism of the integration of multiple risk factors (hyperglycemia, hyperinsulinemia, and high plasma cytokine levels) in the metabolic syndrome in terms of enhanced expression of procoagulant/proinflammatory proteins.

The transcription factor NF-κB is expressed ubiquitously including the liver, and plays a central role in the transcriptional regulation of inflammation-related genes (Barnes & Karin, 1997). During the inflammation/infection, proinflammatory cytokines activate NF-κB in hepatocytes, causing the immediate expression of acute phase proteins such as CRP and SAA, procoagulants such as fibrinogen and factor VIII, and fibrinolysis inhibitor PAI-1 (Chamolstad et al., 2000; Bing, Huang, & Liao, 2000; Fuller & Zhang, 2000; Begbie et al., 2000; Ruan et al., 2001). These proteins may play a beneficial role in the acute phase of inflammation (Gabay & Kushner, 1999), but chronic overproduction is known to cause hypercoagulability with resultant thrombotic disorders and/or atherosclerosis. Furthermore, recent studies suggest that adipose tissue produces TNF-α and other cytokines (Matsuzawa, Funahashi, & Nakamura, 1999), which also stimulate the NF-κB-dependent transcription. Our present data extend the lines of evidence, suggesting that high glucose is also responsible for the activation of the NF-κB-mediated inflammatory process in the liver. The effect seems to be promoter-specific and not caused by hyperosmolality (Loitsch et al., 2000; Takeda et al., 2001), because no increase was observed in RSV promoter-mediated transcription, and the effect was not caused by metabolically inactive L-glucose.

The effect of high glucose alone on NF-κB-dependent transcription was relatively weak in the absence of insulin. On the other hand, significant augmentation was observed with insulin, mimicking the condition in which the combination of high glucose (hyperglycemia) and high insulin concentration (hyperinsulinemia) causes a higher risk in obese patients with diabetes mellitus. Moreover, further activation of NF-κB-dependent transcription was observed with proinflammatory cytokines (IL-1β, TNF-α), raising the possibility that NF-κB may play a pivotal role in the integration of multiple risk factors seen in the metabolic syndrome. Recent clinical data clearly show that the constellation of risk factors such as hyperglycemia, hyperinsulinemia, and high plasma cytokine levels produces an increase in plasma PAI-1 and fibrinogen, causing enhanced coagulability and impaired fibrinolysis, with
resultant increase in the risk of cardiovascular events (Schneider, Nordt, & Sobel, 1993; Pandolfi et al., 2001). Our data also show the increased promoter activity of fibrinogen and PAI-1 genes by glucose, in accordance with the increased plasma level of these proteins and hypercoagulability in patients with uncontrolled diabetes (Bruno et al., 1996; Festa et al., 1999).

The precise mechanism whereby high glucose activates the NF-κB pathway is not completely understood. Chronic hyperglycemia causes the production of advanced glycation end product (AGE), which is known to generate ROS with subsequent activation of NF-κB (Mohamed et al., 1999; Bierhaus et al., 2001). However, in this study, the effect of high glucose was observed as early as 6 h, suggesting the involvement of AGE to be unlikely. Previous studies suggest the involvement of protein kinase C (PKC) in high glucose-induced NF-κB activation in vascular endothelial or smooth muscle cells (Pieper & Riazul, 1997; Yerneni et al., 1999). In this study, we found that PDTC completely eliminated the effect of glucose/insulin, and markedly attenuated the effect of TNF-α. PDTC is usually recognized as an NF-κB inhibitor, but is known to exert this effect via its antioxidant properties. Since a recent study suggests that hyperglycemia acutely produces oxidative stress (Nishikawa et al., 2000), we hypothesize that high glucose enhances the production of ROS in hepatocytes, which in turn activates NF-κB-dependent transcription possibly through PKC. The suppressive effect of PDTC on TNF-α-mediated NF-κB activation is also explained by a recent report that TNF-α generates ROS by activating NADPH oxidase (Li et al., 2002).

In conclusion, our in vitro data strongly support the clinical hypothesis that, besides the microvascular injury as a complication of chronic hyperglycemia, short-term high plasma glucose, alone or in combination with other risk factors, causes the accumulated activation of NF-κB-mediated transcription with a subsequent increase in the risk of thrombotic vascular events.
References


Biofactors 10, 157-167.
Figure legends

Fig. 1. Expression of PAI-1, fibrinogen (Fib), NF-κB p50, p65, and insulin receptor (IR) mRNAs analyzed by RT-PCR in HuH7NF cells. The figure shows photographs of the ethidium bromide-stained products using agarose gel electrophoresis. cDNA produced from an RT reaction using total RNA from HuH7NF cells was amplified by PCR with pairs of oligonucleotide primers specific for each mRNA. No band was amplified in the same reaction without reverse transcriptase (not shown). MW, molecular weight marker.

Fig. 2. Effects of extracellular glucose concentration on the NF-κB-dependent transcription in HuH7NF cells. A. Cells were treated with medium containing the indicated concentration of glucose with or without insulin (1 nM) for 6 h, and the changes in the promoter activity were determined by luciferase assay. *P<.05 vs. value at 3 mM. B. Cell were treated with medium containing 3 or 24 mM glucose with vehicle, insulin (1 nM), or IGF-I (100 nM, PeproTech) for 6 h, and the changes in the cellular protein content per well were determined by a protein assay.

Fig. 3. Time-course effect of high extracellular glucose concentration on the NF-κB-dependent transcription in HuH7NF cells. Cells were treated with medium containing 24 mM of glucose with insulin for 3 to 24 h, and the changes in the promoter activity were determined by luciferase assay. *P<.05 vs. value at time zero.

Fig. 4. The specificity of the effect of high glucose in HuH7 cells. HuH7NF cells were cultured with two different concentrations of D- or L-glucose (3 or 24 mM) for 6 h (left, and middle). Alternatively, HuH7 cells transfected transiently with RSV-luciferase reporter plasmid were cultured with D-glucose (3 or 24 mM) for 6 h (right). The changes in the promoter activity were determined by luciferase assay. *P<.05 vs. value at 3 mM. N.S., not significant.

Fig. 5. Effect of high glucose on the DNA binding of NF-κB. HuH7NF cells were treated
for 22 h with DMEM containing 3 mM glucose, and then incubated with DMEM containing either 3 or 24 mM glucose for 2 h. The cells were harvested and the extracted nuclear protein was used for EMSA analysis. LG, low glucose; HG, high glucose.

Fig. 6. The combined effects of high extracellular glucose and proinflammatory cytokines on the NF-κB-dependent transcription in HuH7NF cells. HuH7NF cells were treated with high glucose (24 mM) for 6 h and/or with human IL-1β (100 pM; left) or human TNF-α (100 pM; right) for 12 h, and the changes in the promoter activity were determined by luciferase assay. *P<.05 vs. cytokine alone.

Fig. 7. The effect of high extracellular glucose concentration on the promoter activities of the coagulation/fibrinolysis-related proteins. HuH7 cells were transiently transfected with each promoter-luciferase construct, and then treated with high glucose (24 mM) for 6 h. The changes in each promoter activity were determined by luciferase assay. *P<.05 vs. value at 3 mM.

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Fig. 9. Schematic representation of the hypothesis based on the present data showing the molecular background of the metabolic syndrome (multiple risk factor syndrome). Hyperglycemia alone, or in combination with hyperinsulinemia and/or high proinflammatory cytokines enhances NF-κB-dependent transcription in the liver (and possibly in the arterial wall), with the subsequent increase in the production of pro-coagulant/anti-fibrinolytic proteins such as fibrinogen or PAI-1, both of which promote thrombosis and/or atherosclerosis.
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