High glucose elevates c-fos and c-jun transcripts and proteins in mesangial cell cultures

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High glucose elevates c-fos and c-jun transcripts and proteins in mesangial cells. It has been previously shown that rat glomerular mesangial cells synthesized increased amounts of fibronectin, laminin, and type IV collagen when grown in medium containing 30 mM glucose. High glucose exerted its effect at the mRNA level since transcripts for all three extracellular matrix (ECM) proteins were similarly elevated. High glucose appeared to exert its effect on ECM mRNA levels through protein kinase C activation. Using quantitative reverse transcription (RT) PCR, we now report that mRNA levels for c-fos and c-jun were increased approximately twofold after treatment with high glucose. The fos levels were elevated 15 minutes after addition of high glucose and were maintained elevated through 30 minutes; by one hour mRNA levels for fos returned to control levels. c-jun, on the other hand, was increased at two hours and remained elevated at 24 and 48 hours. Fibronectin mRNA levels were increased three- to fourfold at 24 and 48 hours. Immunofluorescence studies with polyclonal antibodies to c-fos and c-jun revealed that high glucose treatment for four hours increased nuclear staining intensity two- to threefold for both proteins. Nuclear staining for fos returned to control levels by 24 hours while staining for jun remained elevated. These determinations were made on images obtained on a confocal laser scanning microscope. Thus, high glucose may effect gene expression of ECM proteins by elevating the transcription factors c-fos and c-jun which complex with one another to form activator protein 1 (AP-1).

Diffuse thickening of basement membranes is the most consistent morphologic feature in diabetes mellitus. The thickening is due to increased accumulation of ECM proteins and is evident in many capillary beds in the body including the kidney, retina and nerve giving rise to the characteristic microangiopathy of these organs [1]. In the kidney, it is the expansion of the glomerular mesangium with increased amounts of ECM material that is most closely associated with loss of filtration function [2].

Although considerable controversy still exists concerning the relevant factors responsible for the development of the complications of diabetes, a recent report from the Diabetes Control and Complications Trial has revealed the importance of strict metabolic control in delaying the onset and slowing the progression of the complications of diabetes [3]. In addition, studies performed on cultured endothelial cells and mesangial cells have shown that high glucose per se results in increased synthesis of ECM proteins [4—8]. High glucose appears to exert its effect on cultured cells by activating protein kinase C [9, 10]. The role of protein kinase C activation in this response to high glucose was confirmed by employing either synthetic diacylglycerols which permeate the cell membrane or tumor promoting phorbol esters to activate protein kinase C directly and stimulate the physiologic response (which in this case is increased ECM mRNA levels) [9].

Since protein kinase C is activated in response to high glucose, it is attractive to speculate that ECM genes contain consensus sequences in their promoter regions that interact with transcription factors modulated by protein kinase C. One such factor is known as activator protein 1 (AP-1) [11]. AP-1 has been identified as the transcriptional product of several of the jun and fos proto-oncogene families [11]. Fos and jun associate with each other to generate stable heterodimers which have high DNA binding activity. AP-1 mediates transcriptional induction in response to activators of protein kinase C, such as the phorbol esters 12-O-tetradecanoylphorbol 13-acetate (TPA) and phorbol myristate acetate (PMA), through binding to a conserved DNA element (5'TGACTCA-3') known as the TPA-responsive element (TRE) [11]. The fibronectin and laminin B1 gene contain consensus sequences in its promoter which binds AP-1 [12, 13]. The results reported here show that high glucose results in a rapid transient increase in c-fos mRNA levels followed by a more sustained increase in c-jun mRNA. There are also corresponding increases in c-fos and c-jun proteins. Thus, the transcription factor AP-1 may mediate induction of ECM genes following activation of protein kinase C after treatment with high glucose.

Methods

Cell culture

Rat glomerular mesangial cells were isolated and grown in homogeneous culture as previously described [14]. Mesangial cells were identified by ultrastructural, biochemical, and immunofluorescence techniques [15]. Cells were cultured in RPMI 1640 tissue culture medium (Irvine Scientific Inc., Irvine, California, USA) containing 20% fetal calf serum (Irvine Scientific Inc.) plus antibiotics and antimycotics. All experiments were performed on cells between the 10th and 20th passages.
Kreisberg et al.: High glucose and ECM proteins

RNA isolation, RT-PCR and product quantitation
Mesangial cells were grown to confluence and allowed to sit for three days without a media change. Glucose was added to achieve a final glucose concentration of 30 mM (high glucose). Control cells were maintained in RPMI 1640 with serum (10 mM glucose, normal glucose). After 15 and 30 minutes, and one, two, 24 and 48 hours of high glucose treatment, total cellular RNA was isolated by the single step method of Chomzynski and Sacchi [16]. The RT-PCR procedure was adapted from Chen et al [17, 18]. Approximately 1 to 2 μg of total RNA was used to obtain c-fos, c-jun, fibronectin and aldolase cDNAs for use in PCR amplification. The c-fos and c-jun primers were obtained from Clontech Laboratories Inc. (Palo Alto, California, USA), the fibronectin primers were synthesized on an ABI 380B DNA synthesizer (Applied Biosystems, Foster City, California, USA), and the aldolase primers were supplied by Dr. Robert Kiebe. Aldolase levels are assumed to be constant under experimental and control conditions and are used as a correction factor to account for possible errors in RNA quantitation and/or pipetting of RNA samples. Since the same primer set is used for this comparison the efficiencies of the primers employed are identical.

The c-fos primers employed were 5' -CAGTCTGCTGCATA GAAAGGAAACC-3' (anti-sense primer) and 5' -GAGCTGACA GATACTCCAAGCG-3' (sense primer) which amplified a 432 bp PCR product. The c-jun primers were 5' -CGCAAACTTCTCAGCTATTGG-3' and a sense primer located in Exon 1-1 FN-159-5'TTTTGACAACGGGAAGCAT TATCAGATAAA-3' which amplified a 410 bp PCR product. Aldolase primers of 5'-ATTCTGCTGGCAGATGACTGCCATAA-3' (antisense primer) and 5'-TCATCCTGCTCCTGAGACACT CTA-3' (sense primer) yielded a 314 bp PCR product which was used as a housekeeping gene.

The c-fos, c-jun and fibronectin primers used a 60°C annealing temperature while aldolase was annealed at 58°C. PCR was performed with 32P dCTP added to the dNTP reaction mixture and the random-labeled product was sampled at numerous cycles. PCR products were electrophoresed on 5% polyacrylamide gels, visualized by ethidium bromide staining and then excised from the gels. The radioactivity in each band was determined by scintillation counting. Samples were obtained at PCR cycle numbers within the exponential range of amplification (32 to 36 in the case of c-fos; 24 to 28 for c-jun, 16 to 20 for fibronectin, and 28 to 32 for aldolase; Fig. 1). Verification of products was accomplished by observation of the expected size PCR product after electrophoresis and ethidium bromide staining (Fig. 2) and sequencing. To check for specificity, controls were performed by omission of the antisense and sense primers and the positive control cDNA template (Fig. 2).

The RT-PCR products were processed as follows for sequencing. The products were electrophoresed through a 2% agarose gel and stained with ethidium bromide. The gel containing the appropriate PCR fragments was excised and the DNA purified using a gene clean kit (Prep-A-Gen, DNA Purification Matrix, BioRad Hercules, California, USA). The purified fragments were further subjected to asymmetric PCR...
by limiting the concentration of one of the primers. The asymmetric PCR product was sequenced by the chain termination method [19] with Sequitherm thermostable DNA polymerase (Epicentre Technologies, Madison, Wisconsin, USA) using the primers that were limited during the asymmetric PCR.

Determination of c-fos and c-jun protein by immunofluorescence

Rat mesangial cells were plated onto chamber slides and grown to 60% confluence. After rinsing with RPMI 1640 tissue culture medium, the cells were incubated 24 hours without serum and treated for 2, 4 and 24 hours in either RPMI 1640 with 10 mM glucose (normal glucose) or RPMI 1640 with 30 mM glucose (high glucose). Cells were rinsed and fixed for 10 minutes at room temperature with 3.7% paraformaldehyde in PBS. After rinsing in Tris-HCl buffered saline, pH 7.5 (TBS) containing 0.2% saponin (TBS-saponin) the cells were blocked in TBS-saponin with 5% Carnation milk (TBS-saponin-milk) for one hour. The cells were stained using rabbit polyclonal antibodies to c-fos and c-jun (Oncogene Science, Uniondale, New York, USA) 1:25 dilution overnight at 4°C in TBS-saponin-milk. For a control, preimmune serum was used in place of the primary antibody. After washing three times 15 minutes each in TBS-saponin, the cells were incubated four hours at room temperature with a biotinylated goat anti-rabbit IgG secondary antibody. After washing, the cells were treated in the dark at room temperature for one hour with avidin fluorescein (25 μg/ml). Cells were mounted with crystal mount. The degree of staining was assessed both semi quantitatively and quantitatively. The semiquantitative evaluation was performed on coded slides viewed on a Zeiss epifluorescence microscope. The degree of staining was scored on a 0 to 4+ scale. Approximately 100 cells under each condition were evaluated. For quantitative analysis cells were viewed under a Zeiss confocal laser scanning microscope equipped with an imaging analysis system. Nuclear fluorescence staining intensity was determined by IMAGE, an image processing and analysis computer program [10].

Results

We reported previously that high glucose (30 mM) activated protein kinase C as measured by a translocation of Ca++/phospholipid dependent kinase activity from the cytosolic to
the particulate cellular fraction [8, 9]. Control cells were maintained in 10 mM glucose. Although this concentration of glucose is twice that of plasma, this is the minimal amount of glucose that mesangial cells need to propagate [6, 7]. In addition to activation of protein kinase C, mesangial cells treated with high glucose responded by increasing transcripts and protein synthetic rates for the ECM proteins fibronectin, laminin, and type IV collagen [7]. This effect of high glucose was independent of glucose’s effect on raising the medium osmolality [7]. The growth rate of mesangial cells was unaffected by raising medium glucose concentrations to 30 mM [20].

Since the AP-1 transcription factor complex has been implicated as a key molecule in cell signal transduction in response to a variety of growth factors and hormones which exert their effect via activation of protein kinase C [21—26], we wanted to determine whether high glucose treatment affected the members of the AP-1 complex, c-fos and c-jun mRNA and protein levels. The PCR products generated were of the expected size after electrophoresis and ethidium bromide staining (Fig. 2). In addition, no products were generated if either the antisense or sense primers were deleted (Fig. 2). Likewise, omission of the template for the positive control resulted in no product generated. Sequencing these products matched sequences published for rat c-jun, c-fos, fibronectin, and aldolase.

The PCR products generated were analyzed only during the exponential phase of the amplification (Fig. 1). We found that high glucose resulted in a 1.6-fold elevation in c-fos mRNA levels after 15 and 30 minutes (Figs. 3 and 4). fos mRNA levels returned to control values by one hour. jun, on the other hand, was elevated twofold at two hours and remained elevated after 24 hours of high glucose (Figs. 3 and 4). In another experiment a time course was carried out through 48 hours. We found that c-fos levels were elevated threefold at 30 minutes and returned to control values by one hour (data not shown). c-fos remained at control levels at 48 hours while c-jun was elevated threefold after 24 hours and remained elevated approximately threefold after 48 hours of high glucose (normal glucose = 2540 ± 405 cpm vs. high glucose = 7359 ± 971 cpm after normalization with aldolase; cpm obtained from exponential part of amplification). Fibronectin mRNA was increased three- to fourfold after 24 and 48 hours of high glucose (Fig. 4).

Next, we wanted to examine whether there were corresponding elevations in c-fos and c-jun proteins. Since these are nuclear proteins, we performed immunofluorescence staining with polyclonal antibodies and analyzed them semiquantitatively on coded slides under a Zeiss epifluorescence photomicroscope and quantitatively on a confocal laser scanning microscope equipped with an image analyzing system. Cells were fixed and stained after 2, 4 and 24 hours of high glucose. Semiquantitative analyses revealed 3+ staining for c-fos after four hours of high glucose treatment. Staining intensity returned to control levels by 24 hours. c-jun protein was increased at four hours with a score of 3+ and remained elevated at 2+ levels at 24 hours. The quantitative assessment largely confirmed the semiquantitative analysis. This analysis revealed that c-fos and c-jun increased nuclear staining for both proteins (Table 1 and Fig. 5). High glucose treatment for four hours resulted in a twofold elevation in c-fos nuclear staining intensity (Table 1 and Fig. 5). By 24 hours the degree of staining returned to that seen in cells maintained in normal glucose (Table 1 and Fig. 5).

Fig. 3. Comparison graphs of c-fos and c-jun mRNA levels after exposure to high glucose versus normal glucose. A. c-fos expression after 30 minutes of high glucose; B. c-jun expression after two hours of high glucose. Aliquots of PCR products were collected at the cycle numbers indicated, separated by polyacrylamide gel electrophoresis, excised following staining with ethidium bromide and counted.

Discussion

The results of the studies presented here by quantitative RT-PCR showed that high glucose resulted in a rapid, transient increase in c-fos mRNA followed by a sustained increase in c-jun and fibronectin mRNA in mesangial cell cultures. This was followed by a transient increase in c-fos protein and a sustained elevation in c-jun. A similar transient elevation in
Kreisberg et al.: High glucose and ECM proteins

Fig. 4. The effect of 30 mM glucose on c-fos, c-jun and fibronectin mRNAs. Total RNA was reverse transcribed and PCR was performed as detailed in methods. c-fos was elevated at 15 and 30 minutes following addition of 30 mM glucose compared to 10 mM glucose, while c-jun was elevated at two hours after addition of high glucose and remained elevated after 24 hours. Fibronectin mRNA levels were elevated approximately fourfold after 24 hours and remained elevated (3-fold) at 48 hours. Comparisons between 10 and 30 mM glucose were done on PCR products obtained at the same cycle number on the exponential portion of the amplification curve. Results are presented after normalization against aldolase and represent means ± SD. N = 4; P < 0.05 by one way analysis of variance for 15 and 30 mins high glucose versus normal glucose c-fos mRNA, for 2 and 24 hours high glucose versus normal glucose c-jun mRNA, and for 24 and 48 hours high glucose versus normal glucose fibronectin mRNA.

Table 1. Quantitative confocal microscopy

<table>
<thead>
<tr>
<th>Condition</th>
<th>c-fos</th>
<th>c-jun</th>
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<tbody>
<tr>
<td>Normal glucose</td>
<td>72 ± 8</td>
<td>84 ± 4</td>
</tr>
<tr>
<td>4 Hrs high glucose</td>
<td>118 ± 8*</td>
<td>226 ± 11*</td>
</tr>
<tr>
<td>24 Hrs high glucose</td>
<td>63 ± 4</td>
<td>155 ± 4*</td>
</tr>
</tbody>
</table>

The images analyzed here are those shown in Figure 5. Results are expressed as mean ± SD. Similar results were obtained on a total of 12 additional cells in each group that were not photographed.

* P < 0.05 by one way analysis of variance

activation has also been observed with phorbol esters and other activators of protein kinase C [11]. The transient nature of the c-fos mRNA elevation is most likely due to the short half-life of its protein and mRNA as well as repression of the fos promoter by elevated fos protein levels [11, 21]. Although the increases in fos and jun mRNA levels are modest (that is, 2-fold or less), we believe them to be significant for two reasons: (1.) because the previously reported increases in fibronectin, laminin, and type IV collagen mRNAs seen after high glucose treatment were also approximately twofold, and (2.) the nature of the disease process (that is, diabetic microangiopathy) which takes 10 to 15 years to develop. Therefore, small changes over the course of many years would be expected to lead to thickening of vascular basement membranes and loss of organ function.

Mesangial cells like other cell types affected by high glucose (for example, endothelial cells and nerve cells) display a facilitative diffusion transport system for glucose [20]. Saturation of glucose uptake in mesangial cells occurs at a substrate concentration of 30 to 35 mM, and therefore, intracellular glucose concentrations would reach the levels achieved in diabetes. There are increases in the de novo synthesis of diacylglycerol with activation of protein kinase C in mesangial cells and isolated glomeruli treated with high glucose in vitro as well as in glomeruli isolated from diabetic rats [9, 10, 22]. The importance of protein kinase C activation in the observed increases in fibronectin, laminin and type IV collagen mRNA levels was demonstrated with agents such as oleoyl acetyl glycerol and phorbol tumor promoters which activate protein kinase C directly. These agents similarly increased ECM mRNA levels [10].

In many instances, gene regulation in higher eukaryotes is controlled at the transcriptional level. Transcription of class II genes requires in addition to RNA polymerase II both general
and promoter-specific transcription factors [23]. The promoter-specific transcription factors function by binding to specific sequence elements within the promoter region. One such transcription factor known as AP-1 mediates the transcriptional induction in response to activators of protein kinase C such as the phorbol tumor promoters, through binding to a conserved DNA element known as the TPA responsive element (5'-TGACTCA-3') [11]. AP-1 has recently been identified as the transcriptional product of several of the jun and fos proto-oncogene families [11, 24, 25]. fos and jun associate with each other to form stable heterodimers which have high DNA binding activity. Proto-oncogenes have been proposed to play a vital role in cell growth, differentiation and development [26]. Promoter regions of both the fibronectin and laminin B1 gene contain consensus sequences which can bind to AP-1 [12, 13]. The laminin promoter contains TRE [13] while the fibronectin promoter contains a cyclic AMP-responsive element which differs from TRE by just one base pair (5'-TGACGTC-3') [12]. This element can also bind AP-1 [28]. Since fos and jun messages and proteins are elevated in mesangial cells treated with high glucose, it is attractive to speculate that the mechanism by which fibronectin and laminin are elevated in response to high glucose is via binding of the AP-1 complex to specific sequences in the promoter regions of these two ECM genes.

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