Extracellular signal-regulated kinase (ERK) in glucose-induced and endothelin-mediated fibronectin synthesis

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Increased extracellular matrix protein deposition and basement membrane thickening are important features of diabetic angiopathy. One key matrix protein that has been shown to be instrumental in basement membrane thickening is fibronectin (FN). We have previously demonstrated that glucose-induced increased expression of endothelin-1 (ET-1), may in part, be responsible for increased FN expression via nuclear factor-κB (NF-κB) and activating protein (AP-1) activation. The present study was aimed at elucidating the mechanism of ET-1 with respect to mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway activation and glucose-induced FN upregulation. Human endothelial cells were exposed to either low (5 mM) or high (25 mM) glucose levels. Cells in low glucose were also treated with ET-1 peptide (5 nM). In addition, we treated cells exposed to high glucose levels with specific MAPK/ERK inhibitor PD098059 (50 μM), dual ET-receptor antagonist, bosentan (10 μM), and PKC blocker, chelerythrine (1 μM). Following incubation period, RNA and total proteins were extracted for RT-PCR for FN and immunoblot analysis of MAPK/ERK activation. Confocal microscopy was performed for analysis of FN protein and nuclear localization of activated Elk. Electrophoretic mobility shift assay was carried out to detect NF-κB and AP-1 activation. Our data demonstrates that high glucose-induced upregulation of FN messenger RNA and protein levels occur via activation of MAPK/ERK pathway, which was prevented by treatment of cells with bosentan, PD098059 and PKC blocker chelerythrine. Confocal microscopy demonstrated nuclear localization of phospho-Elk protein. Glucose-induced FN expression was also associated with protein kinase C, NF-κB, and AP-1 activation. These results suggested that glucose-induced, ET- and PKC-dependent, upregulation of FN is, in part, mediated via MAPK/ERK activation.

Imbalance between extracellular matrix (ECM) protein production and degradation is an important feature of chronic diabetic complications.[1,2] Such imbalance is integral to a key structural anomaly of the vascular tissue in diabetes, basement membrane thickening. One predominant ECM protein that has been shown to be upregulated in diabetic angiopathy is fibronectin (FN).[3–6] FN is a glycoprotein of 250 kDa, which in addition to being one of the most abundant proteins in the ECM, is also one of the most specialized.[7] FN molecules interact with various matrix proteins and also modulate numerous cellular processes by interacting with cell surface receptors.[7] *In vitro* studies have demonstrated upregulation of FN by high glucose levels in both vascular endothelial cells and mesangial cells.[6,8]

Elaboration of various hyperglycemia-induced vasoactive factors and growth factors is implicated in augmented FN expression.[1,2] Mechanisms of increased ECM protein production may also include activation of mitogen-activated protein kinase (MAPK) pathway.[8] MAP kinase family form a group of serine/threonine kinases that are activated via phosphorylation following stimulation.[9] MAPK family includes extracellular signal-regulation kinase (ERK), c-jun-N-terminal kinase (JNK) and p38 kinase. ERK is one of the best characterized MAPK families. Generally accepted as a proliferation and differentiation MAPK, ERK is activated by many biological and physiochemical stimuli including growth factors, vasoactive factors, and osmotic stresses.[9] Activation of ERK follows a typical three-stage phosphorylation process.[10] Following stimula-

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tion, activated Raf (MAPKKK) phosphorylates MEK1/2 (MAPKK), which subsequently phosphorylates ERK1/2 (MAPK). There are numerous downstream molecules that are activated by phosphorylated ERK1/2 including nuclear factor Elk.[11]

Glucose-induced coactivation of MAPK and protein kinase C (PKC) has been demonstrated in the mesangial cells.[12] Furthermore, both PKC-dependent and -independent MAPK activation has been shown in the kidney.[13] Downstream effect of MAPK activation may also cause activation of transcription factors such as nuclear factor-kappa B (NF-κB) and activating protein-1 (AP-1).[14] We have previously demonstrated that glucose-induced upregulation of FN in endothelial cells is, in part, mediated by endothelin-1 (ET-1) expression and NF-κB and AP-1 activation.[6] In other systems, MAPK pathway-mediated regulation of NF-κB and AP-1 activation has also been demonstrated.[15,16]

In order to gain insight into the mechanistic basis of glucose-induced FN expression, we have evaluated the potential role of MAPK/ERK activation in glucose-induced and ET-1-mediated FN expression. In addition, we have studied a possible crosstalk between MAPK/ERK and PKC, and its relationship to NF-κB and AP-1 activation.

Materials and methods

Endothelial Cells

Human umbilical vein endothelial cells (American Type Culture Collection, Rockville, MD, USA) were cultured as previously described.[6] Subconfluent cells were incubated with either 5 mM D-glucose (control) or 25 mM D-glucose (high glucose; HG). Cells in 5 mM glucose were also treated with 5 nM ET-1 (Peninsula Laboratories, Belmont, CA, USA). In addition, we incubated cells exposed to high and low glucose levels with either 10 μM dual ET receptor antagonist, bosentan (Courtesy of Dr M Clozel/Acetelion Ltd, Allschwill, Switzerland), 50 μM specific MEK1/2 inhibitor PD098059 (New England Biolabs, Inc., Beverly, MA, USA), and 10 μM U0126 (Promega Corporation, Pittsburgh, PA, USA) or 1 μM PKC inhibitor, chelerythrine (Sigma-Aldrich, Ontario, Canada).

Cells were pretreated with inhibitors for 1 h before exposure to high glucose. Concentrations of peptides and inhibitors were in accordance with the manufacturer's recommendations. Following 24-h incubation period, RNA was extracted for FN gene expression analysis by real-time RT-PCR. In addition, total and nuclear proteins were isolated for analysis of MAPK pathway activation and transcription factor activation, respectively. In addition, cells were cultured on coverslips for immunofluorescent analysis and confocal microscopy.

Cell Viability Assay

We used crystal violet assay to measure cell viability and to exclude potential cytotoxic effects of the compounds as described previously with some minor modification.[17] HUVECs were seeded at 2×10^4 cells/well in 96-well microtiter plates. When the cells were confluent, they were treated with various concentrations of PD98059, U0126, bosentan, chelerythrine for 24 h after they underwent 12 h serum-free conditions. Cells were washed with PBS buffer, fixed with methanol for 5 min, and then stained for 10 min with a 0.1% crystal violet solution. Following three washes, the dye was eluted with 0.1 M trisodium citrate in 50% ethanol for 10 min. Optical density at 630 nm was monitored on a microtiter plate reader.

RNA Isolation and Complementary DNA Synthesis

Real-time quantitative RT-PCR for FN gene expression was carried out in LightCycler™ (Roche Diagnostics Canada, Quebec, Canada) using SYBR Green l for detection. PCR reactions were performed essentially as described.[18] The reaction mixture consisted of 10 μl of ReadyMix SYBR™ (Sigma-Aldrich, Canada), 1.6 μl of 25 mM MgCl$_2$, 1 μl of each forward and reverse 10 μM primers (Table 1), 5.4 μl H$_2$O, and 1 μl complementary DNA (cDNA) template. Messenger RNA (mRNA) levels were quantified using the standard curve method. Standard curves were constructed by using serially diluted standard template. The data were normal-

Table 1 Oligonucleotide sequences for real-time RT-PCR

Gene	Sequence (5′→3′)	PCR temperatures[a]	
18S rRNA	GTAACCCGTTGAACCCCATT CCATCCAATCGGTAGTAGCG (Chen *et al*[18])	Denaturation	95°C for 0 s
		Annealing	55°C for 5 s
		Extension	72°C for 9 s
		Signal	80°C for 1 s
FN	GATAAATCAACAGTGGGAGC CCCAGATCATGGAGTCTTTA	Denaturation	95°C for 0 s
		Annealing	50°C for 6 s
		Extension	72°C for 10 s
		Signal	81°C for 1 s

[a]Initial denaturation was carried out at 95°C for 1 min. Ramp rate for all PCR phases was 20°C/s.

ized to 18S ribosomal RNA (rRNA)[19] to account for differences in reverse transcription efficiencies.

Immunofluorescent Microscopy

Endothelial cells were cultured in 12-well plates (Fisher Scientific, Ontario) with coverslips. Subconfluent cells were treated with various stimulators and inhibitors for 24 h as mentioned above. Following treatment, cells were fixed in anhydrous ethanol and probed with either polyclonal rabbit anti-human FN antibody (1:1000; Dako Diagnostics Canada Inc., Ontario, Canada) or polyclonal anti-rabbit Elk antibody (1:100; NEW England BioLabs Ltd, Ontario, Canada). FITC labeled goat anti-rabbit secondary antibody (Vector Laboratories, Ontario, Canada) was used for detection using a confocal microscope equipped with UV and FITC/TRITC filters (Zeiss LSM 410; Carl Zeiss Canada Ltd, Ontario, Canada).

Protein Purification and Western Blot Analysis

Total proteins from endothelial cells were isolated by homogenizing cells in complete RIPA buffer (NaCl 0.877 g, deoxycholate 1 g, 1 M Tris-HCl pH 7.5 5 ml, and 10% sodium dodecyl sulfate 1 ml; volume adjusted to 100 ml using ddH_2O) and protease inhibitor. Total proteins were then quantified by using BCA protein assay kit™ (Pierce Endogen, Rockford, IL, USA). MAPK/ ERK pathway activation was assessed by using polyclonal phospho-Raf antibody (1:1000; NEW England BioLabs Ltd, Ontario, Canada), phospho-MEK1/2 antibody (1:1000; NEW England BioLabs Ltd), and phospho-p44/42 MAPK antibody (1:1000; NEW England BioLabs Ltd). ECL-PLUS Western blotting detection kit (Amersham Parmacia Biotech., Piscataway, NJ, USA) was used for detection.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously.[6] Briefly, NF-κB and AP-1 consensus oligonucleotide DNA probes (Promega, WI, USA) (Table 2) were prepared by end labeling with [γ-^{32}P] ATP (Amersham, Quebec, Canada) using T4 polynucleotide kinase. The probes

Table 2 Oligonucleotide sequences for EMSA

Transcription factor	Oligonucleotide sequence
NF-κB	5′-AGT TGA GGG GAC TTT CCC AGG C-3′ 3′-TCA ACT CCC CTG AAA GGG TCC G-5′
AP-1	5′-CGC TTG ATG AGT CAG CCG GAA-3′ 3′-GCG AAC TAC TCA GTC GGC CTT-5′

were purified by ethanol precipitation and resuspended in 10 mM Tris and 1 mM EDTA (pH 7.6). Amount of 5 μg of nuclear proteins were incubated with 100 000 cpm of ^{32}P-labeled consensus oligonucleotides for 30 min at room temperature. The incubation was carried out in a buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 5% glycerol, 0.05% IGEPAL CA-630, 0.5 mM EDTA, 0.5 mM DTT, and 0.5 μg of poly(dI-dC). Protein-DNA complexes were resolved on a standard 6% (NF-κB) and 4% (AP-1) nondenaturing polyacrylamide gel in 0.5 Tris-boric acid-EDTA running buffer. After 30 min of electrophoresis at 350 V, gels were dried under heated vacuum onto Whatman paper and subjected to autoradiography from overnight to 3 days. Anti-NF-κB (p65) monoclonal antibody and anti-AP-1 (c-Jun) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for the supershift assay. The specificity of binding was further confirmed by incubation with 100-fold unlabeled oligonucleotides. The blots were quantified by densitometry.[6]

Statistical Analysis

The data are expressed as mean$\pm$s.e.m. and were analyzed by ANOVA followed by *post hoc* analysis. Differences were considered significant at values of $P<0.05$.
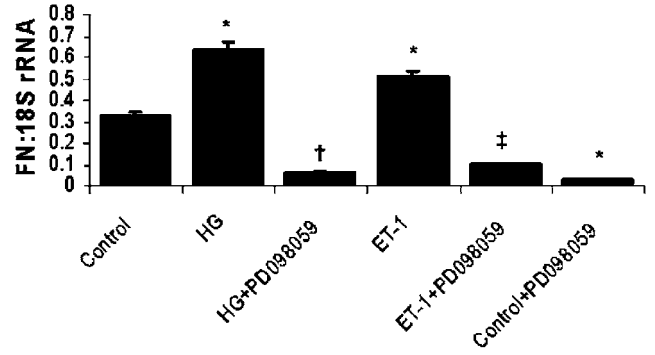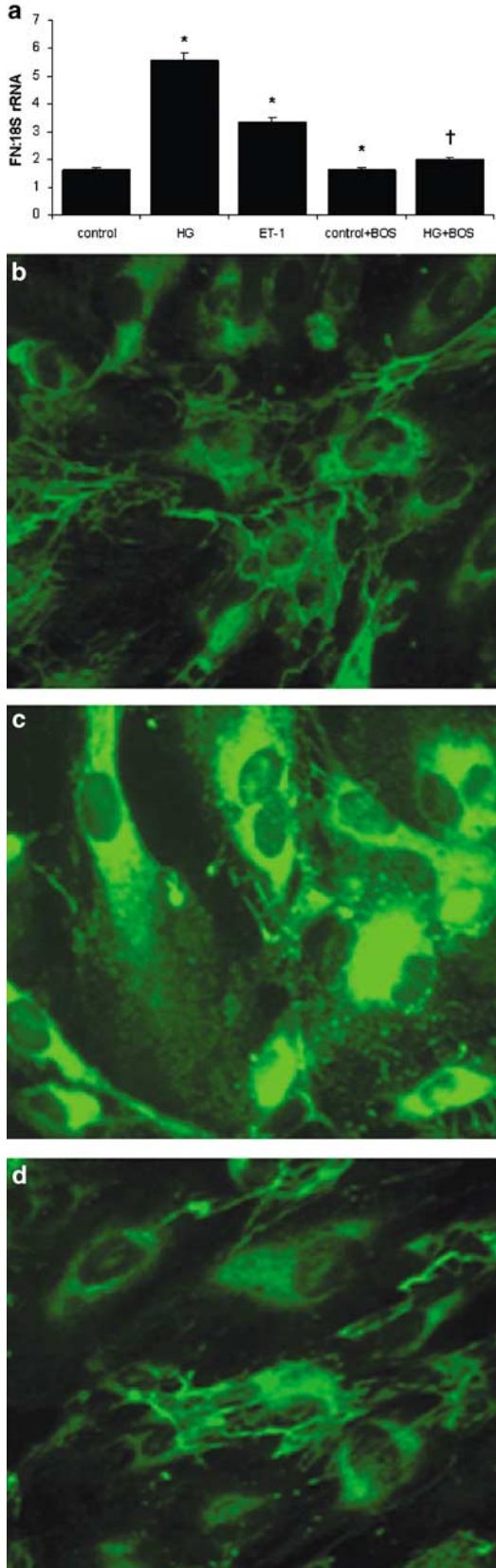
Results

Glucose Causes FN mRNA and Protein Upregulation via ET-1

Exposure of endothelial cells to high glucose levels and ET-1 peptide upregulated FN mRNA levels as determined by real time RT-PCR ($P<0.05$; Figure 1a). These findings are in accordance with our previous studies, which have shown increased expression of FN by high ambient glucose levels and fibrogenic proteins such as ET-1.[6] Incubation of cells exposed to high glucose concentration with dual ET-receptor antagonist, bosentan, abolished glucose-induced FN upregulation ($P<0.05$; Figure 1a). Similar to our previous study, we have demonstrated that a maximal increase of FN mRNA expression is reached after 24 h of exposure and the increase was maintained up to 72 h (data not shown).[6] Hence in subsequent experiments, 24 h exposure in high glucose was performed. In order to elucidate whether alterations in FN mRNA levels coincide with alteration in protein levels, we analyzed endothelial cells with respect to FN protein expression by immunofluorescence microscopy. Our results indicate upregulation of FN protein levels in cells exposed to high glucose concentration (Figure 1b–d). Treatment of cells with ET-1 peptide produced similar results as with exposure to high levels of glucose (data not shown).

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Furthermore, both glucose-induced and basal FN mRNA expression was prevented by dual ET-1 receptor antagonist, bosentan.

Figure 2 Quantitative FN mRNA expression in endothelial cells by real time RT-PCR showed that both high glucose- and ET-1 peptide-mediated FN expression was normalized by inhibitor of MAPK/ERK pathway, PD098059. (ET-1 = 5 nM ET-1 peptide alone; ET-1 + PD098059 = 5 nM ET-1 peptide + 10 μM PD098059; HG + PD098059 = HG + 10 μM PD098059; HG = 25 mM glucose, control + PD098059 = control + 10 μM PD098059; *$P<0.05$ compared to control; †$P<0.05$ compared to ET-1; ‡$P<0.05$ compared to HG; $n=4$/treatment.)
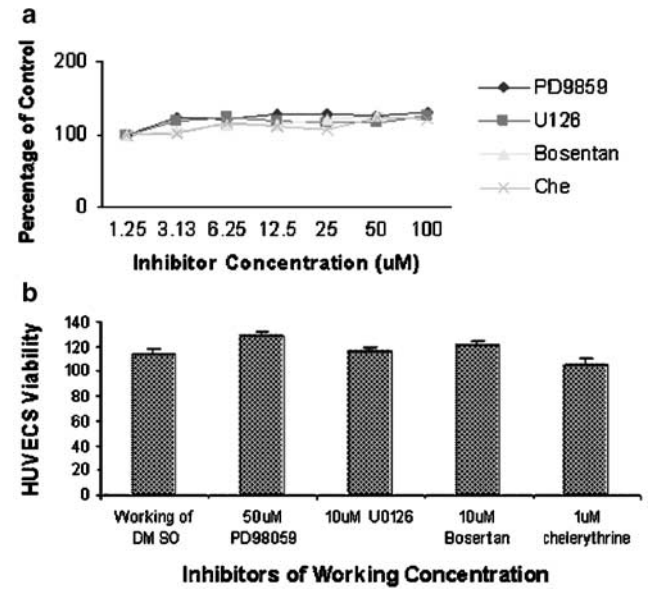
Figure 3 Cell viability analysis (mean $\pm$ s.e.m.) by crystal violet assay: upper panel represents data at various concentrations of different inhibitors used in the experiments, lower panel shows cell viability at the working concentration of the reagents, normalized to DMSO-exposed control. No significant differences were noted.

Figure 1 (**a**) Quantitative FN mRNA expression in endothelial cells exposed to high glucose levels and ET-1 peptide as determined by real-time RT-PCR showing high glucose-induced upregulation of FN mRNA which was normalized by treatment of cells with dual ET receptor antagonist, bosentan. Treatment of cells to ET-1 peptide also increased FN mRNA levels. Representative micrograph of FN protein immunofluorescent analysis in control (**b**), high glucose-treated (**c**), and high glucose with bosentan-treated (**d**) endothelial cells. (ET-1 = 5 nM ET-1 peptide alone; HG = 25 mM glucose, Bos = HG/LG + 10 μM bosentan; *$P<0.05$ compared to control; †$P<0.05$ compared to HG; $n=4$/treatment.)

## Glucose-Induced ET-1-Mediated FN Synthesis is Mediated via MAPK Pathway

In order to elucidate whether glucose- and ET-1-induced FN upregulation is mediated via activation of MAPK/ERK, we evaluated the role of specific MEK inhibitor in cells exposed to high glucose and ET-1 peptide. PD098059 reduced both basal and glucose-induced FN upregulation (Figure 2). U0126, another specific MAPK/ERK inhibitor, also prevented high glucose-induced FN mRNA expression (data not shown). In order to establish that the changes in gene expression are not secondary to cytotoxicity and cell death, we performed crystal violet assay with all inhibitors at various doses including the concentrations used to measure gene expression. No significant effects were seen on cell viability (Figure 3).

Furthermore, activation of MAPK/ERK pathway by high glucose and ET-1 peptide was detected by immunoblot analysis with specific polyclonal phospho-antibodies. High glucose concentration significantly increased activation of Raf, MEK1/2, and ERK1/2 ($P < 0.05$; Figure 4). Similar results were obtained when cells were treated with ET-1 peptide alone. To determine whether glucose-induced activation of MAPK/ERK is mediated via ET-1, we analyzed ERK pathway activation in cells exposed to
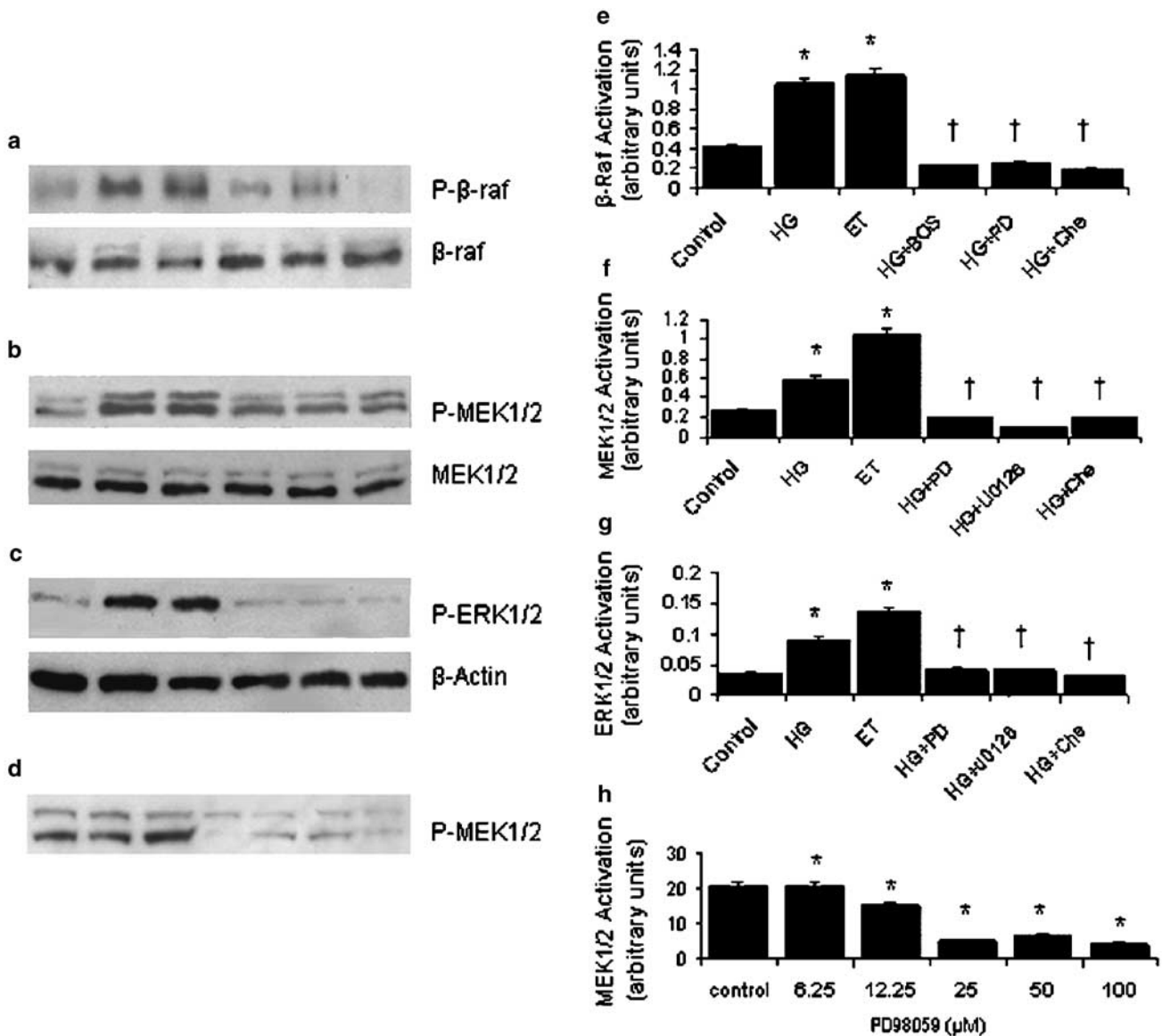

Figure 4 (**a–d**) Representative immunoblot analysis of MAPK/ERK activation showing: (**a**) phospho-Raf, (**b**) phospho-MEK1/2, and (**c**) phospho-ERK1/2. (**d**) Phospho-MEK1/2 showing dose-dependent effect of PD098059 (**e–h**) semi-quantitative analysis of (**a–d**), (ET-1 = 5 nM ET-1 peptide alone; HG = 25 mM glucose, HG + Bos = HG + 10 μM bosentan; HG + PD098059 = HG + 10 μM PD098059; che = HG + chelerythrene 1 μM, *$P < 0.05$ compared to control; †$P < 0.05$ compared to HG; panels **d** and **h** show dose-dependent effect of PD098059, $n = 4$/treatment.) MAPK activation was blocked by MAPK blocker, PKC blocker as well as ET-blocker.
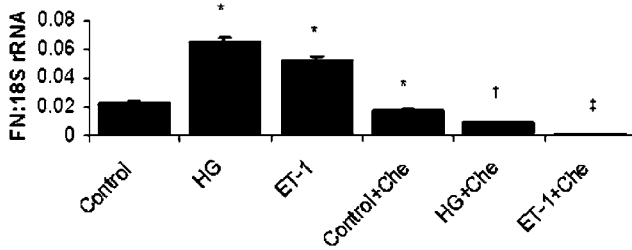
Figure 5 Quantitative mRNA analysis showing interaction between glucose-induced PKC and MAPK/ERK pathway activation in mediating FN mRNA upregulation in endothelial cells. Both high glucose- and ET-1-induced FN mRNA expression was blocked by PKC inhibitor, chelerythrine. (HG = 25 mM glucose; ET-1 = 5 nM ET-1 peptide alone; control + Che = control + 1 μM chelerythrine HG + Che = HG + 1 μM chelerythrine; ET-1 + Che = 5 nM ET-1 peptide + 1 μM chelerythrine; *$P < 0.05$ compared to control; †$P < 0.05$ compared to HG; ‡$P < 0.05$ compared to ET-1; $n = 4$/treatment.)

high glucose and dual ET-receptor antagonist. Inhibition of ET receptor signaling in cells exposed to high glucose completely prevented activation of Raf, MEK1/2, and ERK1/2 ($P < 0.05$; Figure 4). PKC activation has been demonstrated to be an important factor in glucose-induced signal transduction. In addition, a possible crosstalk between PKC and MAPK pathways has been demonstrated in mesangial cells.[12] We found that chelerythrine, a PKC inhibitor, prevented both glucose- and ET-1-induced FN expression (Figure 5). Furthermore, glucose-induced MEK1/2 activation was prevented by chelerythrine, suggesting PKC activation may modulate glucose-induced MAPK phosphorylation (Figure 4).

To further characterize MAPK activation, we have determined localization of Elk, a downstream effector molecule of MAPK/ERK pathway. Confocal microscopy revealed increased nuclear staining of phosphorylated-Elk in cells exposed to ET-1 peptide
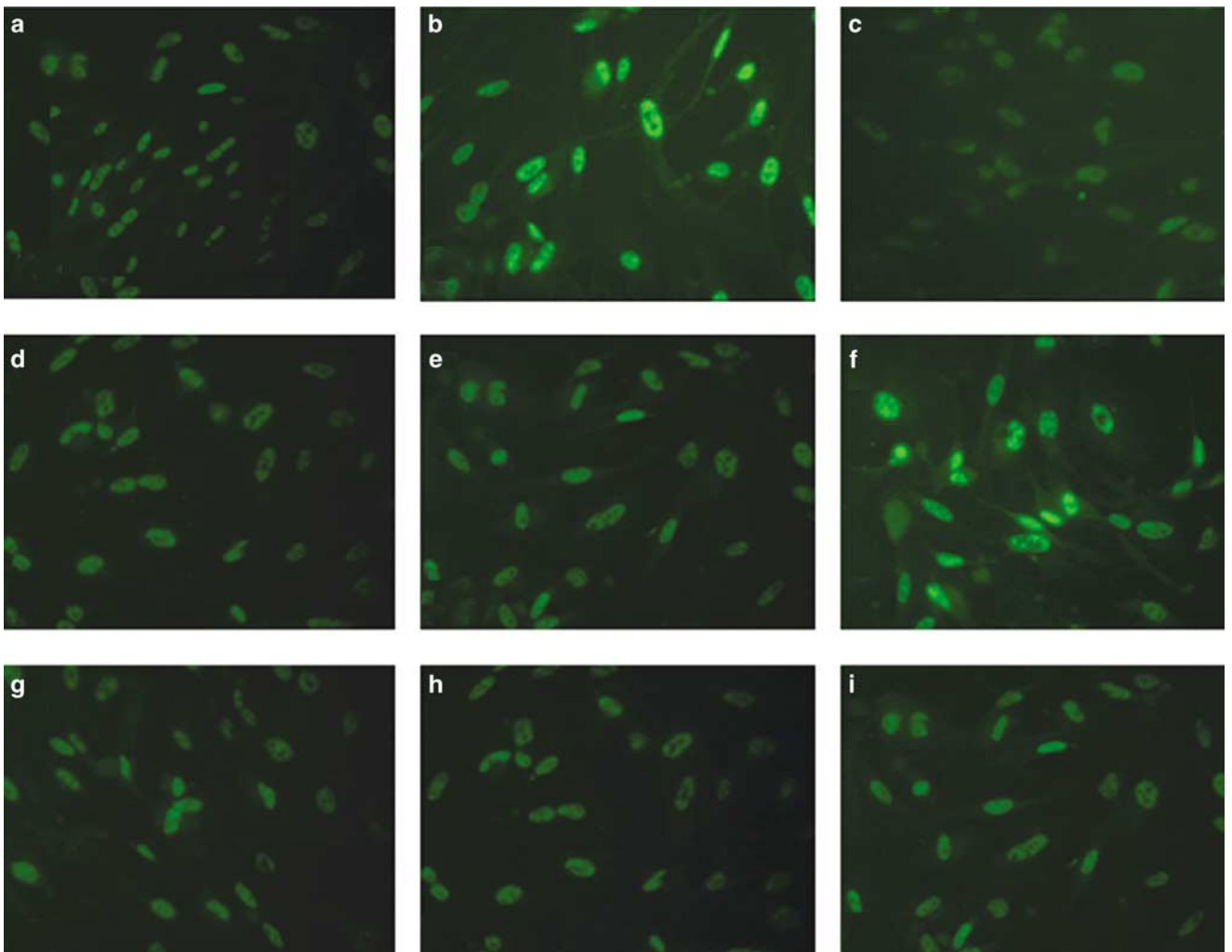
Figure 6 Immunofluorescent analysis of Elk in control endothelial cells (**a**) and cells exposed to (**b**) HG, (**c**) HG + Bosentan, (**d**) HG + PD098059, (**e**) HG + Che, (**f**) ET-1 peptide, (**g**) ET-1 peptide + PD 098059, and (**h**) ET-1 + Che. Both glucose and ET-1 increased immunoreactivity of phospho-Elk, which was attenuated by bosentan, MAPK inhibitor, and PKC inhibitor.

alone or high glucose levels as compared to untreated cells (Figure 6). In addition, inhibitors that prevented glucose-induced FN upregulation and MAPK/ERK activation also reduced nuclear localization of activated Elk (Figure 6).

Transcription Factor Involvement in MAPK-Mediated Signaling of FN Expression

Our next objective was to determine whether glucose-induced and ET-1-mediated MAPK/ERK pathway activation involves transcription factors, NF-κB and AP-1. We performed EMSA in the cells exposed to various conditions as described above. We have previously demonstrated that both NF-κB and AP-1 may be of importance in this scenario.[6,18] EMSA showed glucose-induced activation of both NF-κB and AP-1 in endothelial cells (Figure 7). Inhibition of MAPK pathway

caused marked attenuation of both NF-κB and AP-1 activation (Figure 7). These findings suggest that both transcription factors, NF-κB and AP-1, are predominantly involved in this pathway. Similar activation pattern of transcription factors were also obtained in cells exposed to ET-1 peptide. Dual ET receptor antagonist prevented both NF-κB and AP-1 activation (Figure 7).

Discussion

We have evaluated the role of glucose-induced MAPK/ERK pathway activation in FN expression in endothelial cells exposed to high glucose levels. Our key findings indicate that glucose-induced and ET-1-mediated FN upregulation may involve activation of ERK1/2 pathway. We have also demonstrated
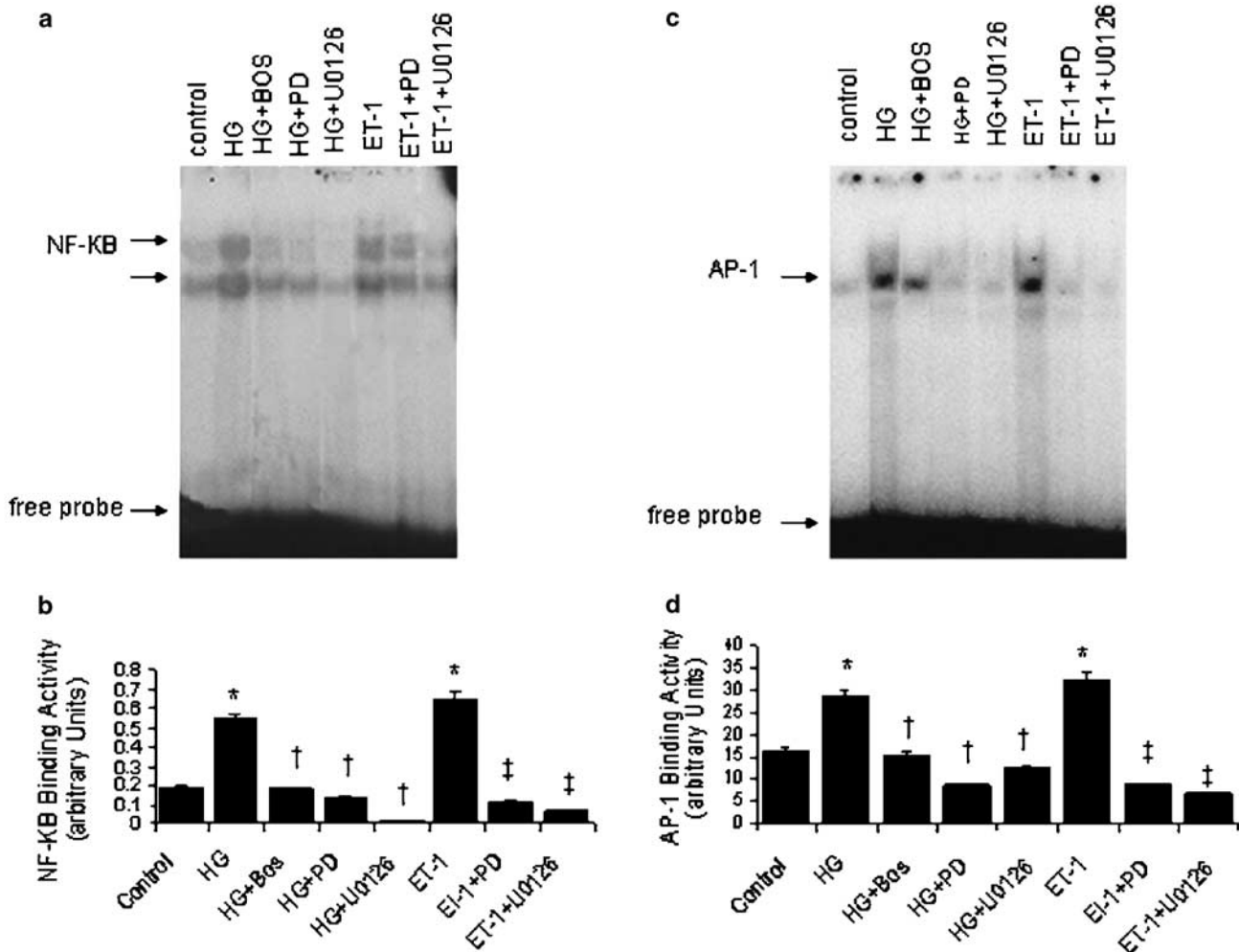

Figure 7 Interaction between high glucose-induced MAPK/ERK activation and activation of transcription factors, NF-κB and AP-1. (**a**) Representative EMSA showing high glucose- and ET-1-mediated activation of NF-κB. (**b**) Semiquantitative analysis of NF-κB activation in nuclear extracts from HUVECs as assessed by densitometry. (**c**) Representative EMSA showing AP-1 activation in HUVECs exposed to high glucose and ET-1 peptide. (**d**) Semiquantitative analysis of AP-1 activation as determined by densitometry. (Lanes: 1, cold; 2, control; 3, HG; 4, HG + 10 μM Bos; 5, HG + 10 μM PD098059; 6, HG + 10 μM U02126; 7, 5 nM ET-1; 8, 5 nM ET-1 + 10 μM PD098059; and 9, 5 nM ET-1 + 10 μM U02126; *$P<0.05$ compared to control; †$P<0.05$ compared to HG; ‡$P<0.05$ compared to ET-1; $n=4$/treatment.)

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that MAPK/ERK pathway may crosstalk with PKC and predominately mediates signal for FN upregulation via NF-κB and AP-1 activation.

Increased expression of ECM proteins such as collagen and FN, leading to basement membrane thickening is the most common pathological feature of chronic diabetic complications.[1–5,18] We have previously demonstrated an important role of hyperglycemia-induced ET-1 expression in increased ECM protein deposition.[4,15,18] We have also shown that hyperhexosemia-induced increased FN mRNA expression can be prevented by bosentan treatment in retinas and kidneys of both diabetic and galactose-fed rats.[4,15] Bosentan treatment also prevented retinal capillary and glomerular basement membrane thickening in these animals. Furthermore, a similar normalization of FN expression by bosentan has been shown in heart tissues of diabetic animals.[20]

The molecular mechanism by which high glucose-induced ET-1 expression leads to increased FN expression and ECM protein deposition remains to be fully elucidated. However, current studies may suggest that both PKC and phosphatidylinositol 3-kinase pathways may converge on MAPKs.[21,22] Furthermore, biochemical anomalies implicated in hyperglycemia-induced vascular dysfunction such as, nonenzymatic glycation, oxidative stress, and alteration in growth factor and vasoactive factor expression are all involved in alteration of ETs and increased ECM deposition.[1,2,23,24] It is being increasingly realized that all of these hyperglycemia-induced secondary factors share the capacity to activate MAPKs. These studies suggest that MAPK activation may represent a common signal transduction of glucose-induced ET-1 mediated ECM protein synthesis. It is interesting to note that, the inhibitors used in this study reduced glucose-induced fibronectin (FN) expression below the level expressed by cells in normal glucose. These are not due to cytotoxicity as demonstrated by crystal violet assay. Furthermore, incubation of cells in normal glucose with the inhibitors shows reduction of basal FN expression. In culture, the endothelial cells normally produce fibronectin. Hence, it is conceptually possible that these pathways may also influence basal FN expression. However, identification of exact mechanisms requires further investigation.

Our data demonstrated several important findings; it appears that PKC is an upstream mediator in this pathway as PKC inhibitor blocked MAPK activation. PKC is a known upregulator of ET-1.[25] On the other hand, ET-1 receptor, being G_q coupled, may activate phospholipase C, increase intracellular calcium and may cause PKC activation.[1,2,26] Hyperglycemia-induced NF-κB activation may also cause upregulation of ET-1.[27] In several systems, ET-1 has been also shown to mediate its effect via MAPK pathway.[28–30] FN gene has both NF-κB and AP-1 binding site on its promoter and may be positively
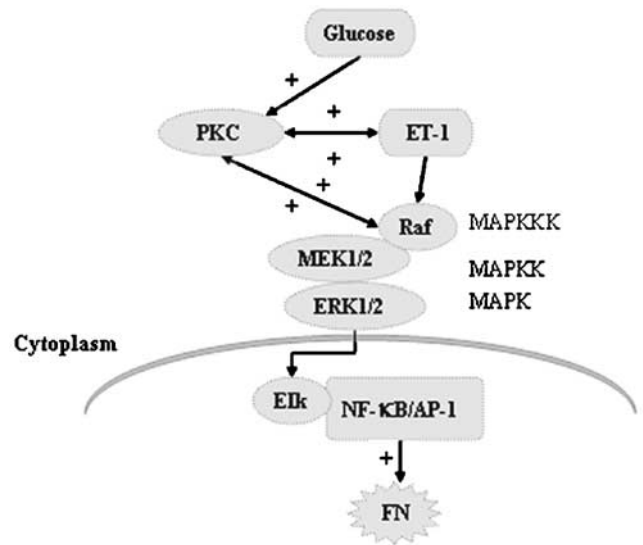
Figure 8 A schematic outline of mechanisms leading to glucose-induced increased fibronectin synthesis in the endothelial cells as demonstrated in this study.

regulated by both transcription factors.[31–35] Data from our present study would suggest that MAPK pathway may upregulate FN via NF-κB and AP-1. However, it is possible that glucose-induced PKC and ET-1 activation may also lead to FN upregulation independent of MAPK activation. A schematic outline of pathways of glucose-induced FN synthesis, as investigated in this study, is outlined in Figure 8.

In summary, we have demonstrated that glucose-induced PKC and ET-1 activation may upregulate FN mRNA and protein expression via MAPK-dependent NF-κB and AP-1 activation. Identification of such pathogenetic mechanism leading to glucose-induced increased ECM protein synthesis is of importance as these may form the basis of new drug targets or novel adjuvant treatment modalities for diabetic complications.

Acknowledgements

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