High Glucose Concentration Decreases Insulin-like Growth Factor Type 1–Mediated Mitogen-Activated Protein Kinase Activation in Bovine Retinal Endothelial Cells

V.A. McBain, M. Robertson, E. Muckersie, J.V. Forrester, and R.M. Knott

Clinical trials have incontrovertibly demonstrated that the onset and progression of diabetic retinopathy (DR) is influenced by the control of glucose levels in patients. In the present study, we examined the effect of glucose concentration on the responsiveness of bovine retinal endothelial cells (BREC) to insulin-like growth factor type 1 (IGF-1). Retinal endothelial cells were isolated from bovine retina and cultured in 5 or 20 mmol/L glucose with or without 100 ng/mL IGF-1. The level of cell growth and p42/44 and p38 mitogen-activated protein kinase (MAPK) activation was determined using the alamarBlue (Serotech) assay and Western blotting, respectively. IGF-1 significantly enhanced cell growth in BREC exposed to 5 mmol/L glucose but not in cells exposed to high glucose concentrations (20 mmol/L). IGF-1 induced a transient activation of p42/44 MAPK, with peak activation at 15 minutes in cells exposed to 5 mmol/L glucose; however, no increase in p42/44 MAPK was evident at the higher glucose concentration of 20 mmol/L. There was no significant change in the level of p38 MAPK during the time period examined when IGF-1 was also present. However, high glucose concentrations alone increased the level of p38 MAPK after 60 minutes and the level of p42/44 MAPK after only 15 minutes exposure in 20 mmol/L glucose. Thus, BREC exposed to high glucose concentrations are not sensitive to IGF-1 and this is due, at least in part, to a reduced activation of the p42/44 MAPK pathway. Furthermore, the presence of IGF-1 appears to exert a protective effect on the cells in high glucose concentration by preventing progression through the cell cycle.

DIABETIC RETINOPATHY (DR) is the most common cause of blindness in the working population of the Western world. Clinical trials have demonstrated a correlation between the incidence and severity of DR in those patients with poor glucose control.[1] Glucose is being increasingly viewed as a mediator of physiological and pathological processes in addition to its role as an essential nutrient. Indeed, the concentration of glucose that the cells are exposed to is fundamental to the ability of cells to proliferate in response to growth factors,[2] which are themselves known to play a central role in the pathology of DR.[3-9]

Intensive insulin therapy is known to slow the progression of retinopathy and reduce the development of proliferative or severe nonproliferative DR.[1] However, once the tissue damage has progressed to overt proliferative retinopathy, the benefits of a tight glucose regimen are not clear and caution in the treatment of patients with proliferative or severe nonproliferative retinopathy with intensive insulin therapy has been suggested.[10] Thus, it seems likely that there are at least 2 distinct phases within retinopathy, the earlier phase being associated more directly with glucose levels and the later stage being driven by events that have resulted from the damage caused by the high concentrations of glucose. The early phase of DR reflects changes in retinal endothelial response resulting from high concentrations of glucose.[11] It is therefore necessary to address issues relating to the anti-angiogenic and/or anti-apoptotic potential of factors that influence retinal endothelial cell growth in the early stages of onset of disease.

Levels of insulin-like growth factor type 1 (IGF-1) are reported to be high in people with diabetes and the clinical evidence suggests that a cause-effect relationship may exist between DR and high IGF-1 levels.[12,13] Uniform populations of high-affinity IGF-1 receptors are found in bovine retinal endothelial cells (BREC) and the addition of IGF-1 to these cultured cells has been shown to induce a 4-fold increase in DNA synthesis[14] and to protect against apoptosis. IGF-1 mediates its effect via the activation of phosphotidyl inositol 3-kinase[15] leading to an increase in the downstream target of the *ras*-mitogen-activated protein kinases (MAPK).[16]

Other significant observations include the linking of intracellular cyclic adenosine monophosphate (camp) with IGF-1 action[17]; consequently IGF-1 action is modified when the concentration of glucose is altered. Furthermore, IGF-1 has been shown to prevent apoptosis in cells exposed to high concentrations of glucose in a rat model.[18] We have therefore examined the response of retinal endothelial cells with respect to cell growth and the activation of p42/44 MAPK and p38 MAPK in euglycemic (5 mmol/L) and hyperglycemic (20 mmol/L) conditions and in response to the presence of IGF-1. The level of 42/44 MAPK was chosen as an indicator of the *ras*-MAPK pathway and p38 MAPK is known to be associated with changes in osmolarity that may result from the incubation of the cells in high glucose concentration. The rationale underlying this approach is that high glucose concentrations decrease endothelial cell growth,[11] which may result in a decreased cell responsiveness to key growth factors. This has implications for wound healing and for the early events associated with the development of microvascular complications of diabetes.

We propose that a glucose-mediated change in retinal endothelial cell response to IGF-1 occurs, which is evident as a lack of sensitivity of BREC to IGF-1 in conditions of high glucose concentration.

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Submitted February 21, 2002; accepted September 19, 2002.

Supported by the Royal National Institute for the Blind.

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0026-0495/03/5205-0045$30.00/0

doi:10.1053/meta.2003.50046

MATERIALS AND METHODS

Cell Culture

Bovine eyes were obtained from a local abattoir and kept on ice until they were used, usually within 2 hours of enucleation. BREC were isolated, as previously described,[19] and cultured in glucose-free Glasgow's Minimal Essential Medium (GMEM; Gibco, BRL, Paisley, UK) supplemented with 5 mmol/L glucose with 5% platelet-deprived serum (PDS). The endothelial cells were grown in 75-cm^2 flasks until they were approximately 80% confluent and then they were either reseeded into microtiter plates for the alamarBlue (Serotech, Oxford, UK) assays, or the test agents were prepared for Western blot analysis.

Cell Growth

The alamarBlue dye incorporates a colourimetric redox growth indicator that reacts to metabolic activity and thus reflects the growth of the cells. BREC were seeded into 96-well microtiter plates at a concentration of 0.05×10^6/mL and incubated for 24 hours. The cells were stepped-down in glucose-free, serum-free medium for approximately 18 hours and then α-D-glucose (5 and 20 mmol/L) was added in the absence and presence of 100 ng/mL IGF-1 supplemented with 1% PDS. The alamarBlue dye was added immediately (10% vol/vol) after the test agents and then the microtiter plates were incubated at 37°C, 5% CO_2 for 30 minutes. The plates were read on an enzyme-linked immunsorbent assay (ELISA) plate reader (Dynatech MR5000, Dynex, West Sussex, UK) at a wavelength of 570 nm to give the t = 0 hour time-point. Subsequent readings were then taken at 6, 24, 48, and 120 hours. A mean value (± SD) for 6 replicate wells was calculated and the t = 0 hour reading was subtracted from the subsequent readings to give cumulative growth. The results are representative of 3 separate experiments.

Western Blotting

BREC were grown in 75-cm^2 flasks until approximately 80% confluent and then stepped-down overnight in glucose-free, serum-free medium. The BREC were then exposed to 5 and 20 mmol/L glucose for 0, 15, 30, and 60 minutes in the absence and presence of 100 ng/mL IGF-1. Cells were also incubated in PD98059 (2 μm; Calbiochem Novabiochem, Nottingham, UK), which inhibits phosphorylation of p42/44 MAPK. This allowed us to determine the specificity of the IGF-1–mediated increased level of p42/44 and p38 MAPK. The assays were also performed in the presence of an equivalent concentration of mannose in order to determine if there were any effects due to the change in osmolarity. The cells were harvested using a lysis buffer (1% sodium dodecyl sulfate [SDS], 1 mmol/L sodium vanadate, and 10 mmol/L Tris HCl pH 7.4), boiled for 5 minutes, and then aspirated. The cell extracts were normalized for total protein using the Coomassie protein assay (Pierce & Warriner UK Ltd, Chester, UK) and 50 μg of each sample was resolved through a 4% to 20% polyacrylamide gradient gel (BioRad Laboratories, Hemel Hempstead, UK). The sample proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham International plc, Little Chalfont, UK) by electroblotting and the membranes were incubated in 5% nonfat milk in TBS-T (tris-buffered saline [TBS] with 0.1% Tween-20) to block nonspecific binding to the membrane. The membranes were incubated with rabbit polyclonal anti-p42/44 or anti-p38, both antibodies being directed against the active portion of the molecule (New England Biolabs, Hertfordshire, UK) at 1:2,500 and 1:500 dilution respectively, followed by swine anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP; Dako Ltd, Cambridge, UK) at 1:3,000 dilution. The blots were developed using the enhanced chemiluminescence method (ECLplus, Amersham Pharmacia Biotech) and then the intensity of signal on the hyperfilm ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK) was determined using the Genesnap/tools gel documentation system from Syngene (Cambridge, UK). The blots were stripped according to the ECLplus protocol and reprobed using mouse monoclonal anti-pERK (Transduction Labs, Lexington, KY) at 1:5,000 dilution followed by the secondary rabbit anti-mouse HRP at 1: 3,000 dilution. This enables the total level of phosphorylated and nonphosphorylated p42/44 and p38 MAPK to be determined. Results are presented as a ratio of p42/44 or p38 activation to extracellular regulated kinase (pERK) levels and are presented as mean values of results obtained from a minimum of 3 different batches of cells. Validation of the method of quantitation included silver staining of the PVDF membrane to determine protein loaded as described for the quantitation of the Hyperfilms. The pERK/protein loading was found to be approximately 1.0 in each case (results not shown), indicating that the pERK level was an accurate internal standard of protein levels.

Statistical Analysis

The statistical significance of the result was determined using the Student's t test or alternatively the Welch's t test when the standard deviations were significantly different. The level of significance is expressed as $P < .05$ or $P < .01$.

RESULTS

Effect of Glucose Concentration on IGF-1–Stimulated Cell Growth

To determine the effect of BREC responsiveness to IGF-1 stimulation, the level of cell growth was determined. BREC growth rose steadily during the first 48 hours of incubation in both 5 and 20 mmol/L glucose and was seen to level off by 120 hours. BREC responded to IGF-1 with a significant increase in cell growth ($P > .05$) (Fig 1A). However, no significant IGF-1–mediated cell growth was observed in cells grown in 20 mmol/L (Fig 1B). Results are presented as the cumulative net growth over the period of observation and are expressed as absorbance at 570 nm.

Time- and Glucose-Dependent Activation of p42/44 MAPK by IGF-1

We then determined whether the altered response of BREC to IGF-1 in high glucose concentrations was in any way reflected by a change in intracellular signaling using a time-dependent analysis of the level of phosphorylated p42/44/MAPK. IGF-1 activated p42/44 MAPK in a time-dependent manner in BREC exposed to 5 mmol/L glucose (Fig 2A) with peak levels occurring 15 minutes after addition of IGF-1 (Fig 2A, lane 2). For BREC exposed to 20 mmol/L glucose, no significant increase in p42/44 MAPK was observed between the 15-, 30-, and 60-minute time-points (Fig 2B). The total level of ERK (ie, phosphorylated and nonphosphorylated) was also determined by Western blotting. The mean data from 3 identical experiments are presented in Fig 2C as a mean of a ratio of p42/44 MAPK/pERK levels. IGF-1–mediated activation of p42/44 MAPK was inhibited with the addition of 2 μm of PD98059 (Calbiochem Novabiochem) (Fig 3).

We then determined the level of phosphorylation of p38 MAPK and investigated whether this contributed to the failure to activate p42/44 MAPK. We found that the level of p38 remained relatively constant throughout the period of investigation in response to IGF-1 when the cells were incubated at either 5 or 20 mmol/L glucose (Fig 4). Furthermore, there was

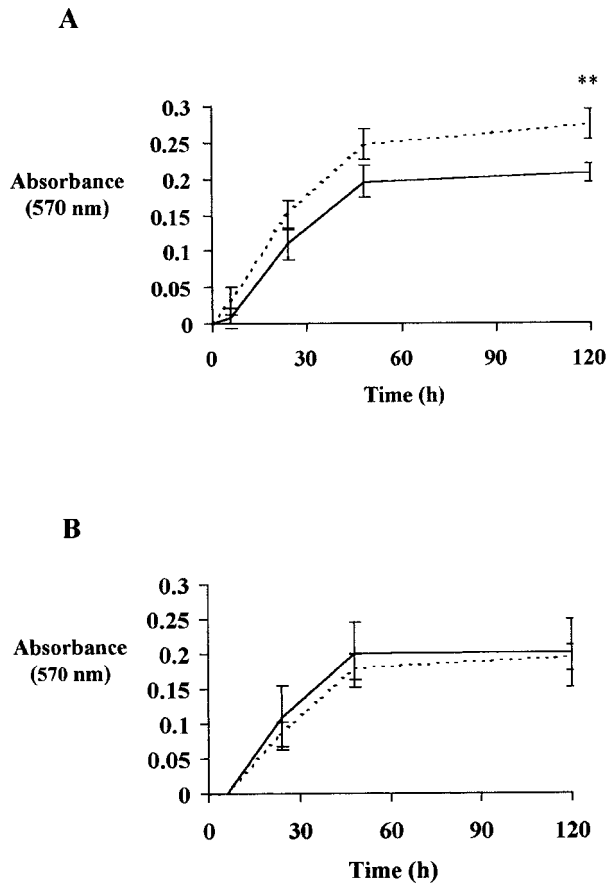

Fig 1. IGF-1–mediated increased cell growth of BREC incubated in 5 mmol/L or 20 mmol/L glucose. IGF-1–treated BREC growth is indicated by the dotted lines. BREC were incubated in (A) 5 or (B) 20 mmol/L glucose over a period of 120 hours excluding a 30-minute incubation in alamarBlue dye, in the absence and presence of 100 ng/mL IGF-1. The results are presented as the cumulative absorbance (nm ± SEM) as indicated by the redox color change of the dye and are representative of 3 separate experiments. IGF-1 induced a significant increase ($P < .01$) in BREC growth incubated in 5 mmol/L glucose, whereas no significant enhancement was observed in BREC growth exposed to 20 mmol/L glucose. **$P < .01$.

no effect on the parameters examined when the cells were incubated in the presence of mannose (results not shown).

Effect of Glucose Concentration of the Activation of p42/44 and p38 MAPK

Results indicated that the level of p42/44 MAPK was elevated after 15 minutes exposure to high glucose concentrations in the absence of IGF-1 and the level of p38 MAPK was elevated after 60 minutes in high glucose (Fig 5). This contrasts markedly with the lack of any significant change in these 2 parameters when the cells are simultaneously exposed to IGF-1.

DISCUSSION

In the present study we investigated the relationship between IGF-1–mediated activation of p42/44 MAPK and glucose concentration. We demonstrated less IGF-1–mediated phosphorylation of p42/44 MAPK in cells cultured in 20 mmol/L glucose compared to 5 mmol/L glucose, indicating reduced responsiveness to IGF-1 and the consequent lack of downstream events that would normally lead to cell proliferation.

The peak of IGF-1–induced activation of p42/44 MAPK occurred at 15 minutes. Interestingly, this did not correlate with any change in the expression of p38 MAPK. The activation of p38 has been shown after exposure to high glucose and/or diabetes after a chronic period of exposure.[20] In this study we demonstrated an increased level of p38 after 60 minutes and an increase in p42/44 after only 15 minutes when IGF-1 is absent. This suggests that while high glucose alone can have detrimental effects on the cells, the presence of the IGF-1 is able to ameliorate the effect. IGF-1 has previously been shown to prevent glucose-induced apoptosis in neuronal cells from diabetic rats.[21] Although this study does not specifically report on the apoptosis of the retinal endothelial cells, the results demonstrate a net decrease in the level of p38 MAPK in the

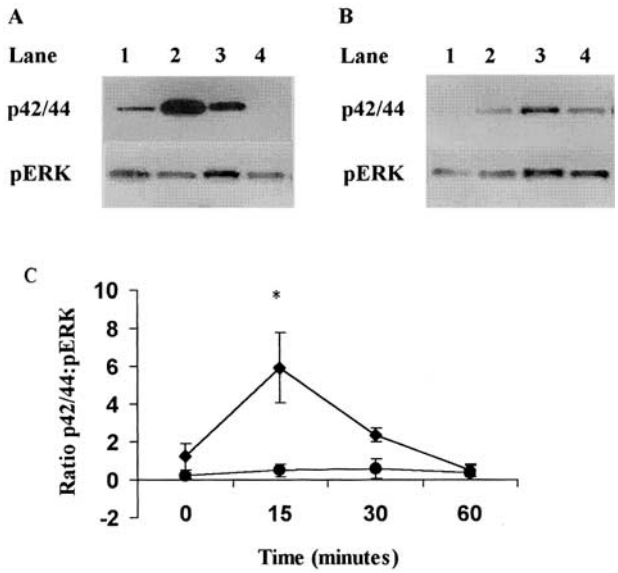

Fig 2. Glucose concentration-dependent activation of p42/44 MAPK following addition of IGF-1. Lane 1, t = 0; lane 2, t = 15 minutes; lane 3, t = 30 minutes; lane 4, t = 60 minutes). (A and B) Representative Western blots from 3 separate experiments illustrate p42/44 activation and pERK levels in BREC following IGF-1 treatment. Addition of IGF-1 to BREC incubated in 5 mmol/L glucose resulted in a significant activation of p42/44 within 15 minutes and that was sustained for a further 15 minutes (A, lanes 2 and 3). However, the application of IGF-1 to BREC cultured in 20 mmol/L glucose did not significantly activate p42/44 within the 60-minute period of investigation (B). (C) Level of p42/44 MAPK in response to IGF-1 in retinal endothelial cells in 5 or 20 mmol/L glucose. BREC were exposed to 100 ng/mL IGF-1 for 15, 30 or 60 minutes in either 5 mmol/L glucose (◆) or 20 mmol/L glucose (●). The results are presented as the mean ratio of p42/44 activation to pERK levels (± SEM), n = 3. The level of p42/44 MAPK was significantly elevated after 15 and 30 minutes exposure to IGF-1 when the cells were incubated in 5mmol/L glucose (*$P < .05$). There was no significant elevation in the level of IGF-1 activation of p42/44 MAPK in retinal endothelial cells cultured in 20 mmol/L.

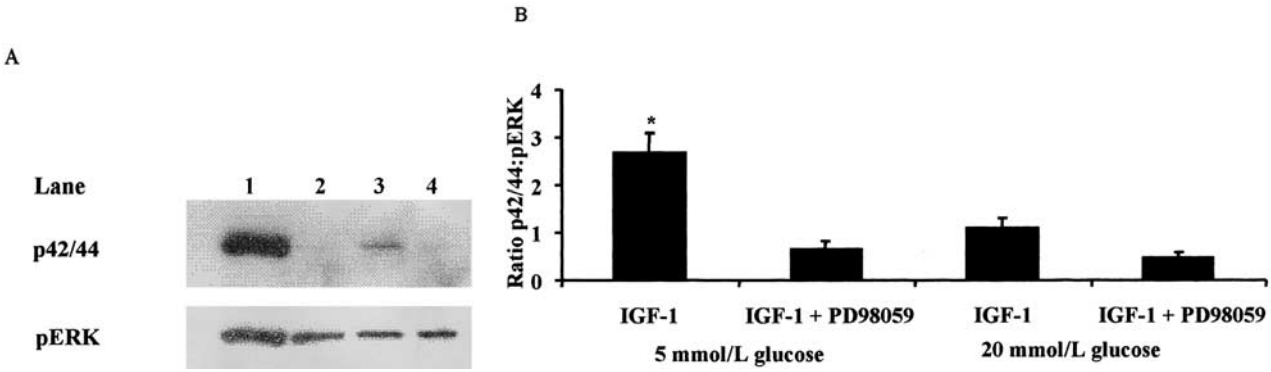

Fig 3. Western blot demonstrating inhibition of IGF-1–mediated activation of p42/44 MAPK with PD98059 (A). (Lane 1, 5 mmol/L + 100 ng/mL IGF-1; lane 2, 5 mmol/L + 100 ng/mL IGF-1 + PD98059; lane 3, 20 mmol/L + 100 ng/mL IGF-1; lane 4, 5 mmol/L + 100 ng/mL IGF-1 + PD98059). BREC were incubated in 5 or 20 mmol/L glucose with IGF-1 (100 ng/mL) for 15 minutes. The results were quantified and are representative of 3 separate Western blots (B). The activation of p42/44 MAPK was reduced when the cells were incubated in the presence of PD98059 in 5mmol/L glucose and was reduced in 20 mmol/L glucose. The activation of 42/44 MAPK was significantly reduced when the cells were incubated in the presence of PD98059 in 5 mmol/L glucose. The mean values of p42/44 MAPK/pERK from 3 separate experiments are presented (± SEM).

presence of IGF-1, which is known to be activated in response to cellular stress.

There is a cell type–specific effect of glucose concentration and the results of this study on retinal endothelial cells should not be extrapolated to other cell types. It has been reported that while selected cell populations, eg, tubulointerstitial fibroblasts, may undergo sustained proliferation in the diabetic environment, most renal cells such as mesangial cells are arrested in the G1 phase of the cell cycle.[22]

It is not yet clear how the high glucose concentration is able to specifically mediate the reduction in IGF-1–mediated p42/44 MAPK while a higher baseline level of activation is evident in the absence of IGF-1. Previous studies have found that high glucose concentrations increase the activation of p42/44 MAPK,[23] while others have shown that nonmetabolizable analogs of glucose also activate MAPK.[24] The changes in MAPK activation in this study were not simply due to the osmotic changes that result from hyperglycemia as indicated by the lack of any effect in the presence of mannose.

Clearly the relationship between glucose concentration and cell growth is closely intertwined because of the essential requirements for increased energy expenditure that is required for cell growth to occur. Further work involving the measurement of protein kinase C (PKC) would extend the present study. PKC has previously been shown to have a negative role in IGF-1–mediated activation of the serine threonine kinase *Akt*, which is an important target of phosphatidylinositide 3-kinase,[25] while others report the activation of p42/44MAPK via PKCmu.[26] Elevation of PKC is known to occur in response to high concentrations of glucose and is believed to play a central role in the development of vascular complications of diabetes.[27] In vitro, it has also been demonstrated that when PKC is inhibited, glucose-dependent decreased growth of retinal endothelial cells is restored.[11]

IGF-1 is a growth factor with known glucose-regulatory and growth-promoting properties. This may account for

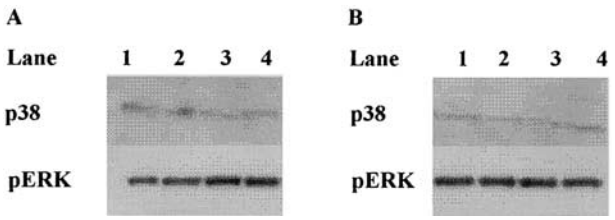

Fig 4. Western blot demonstrating activation of p38 MAPK in 5 or 20 mmol/L glucose in the presence of IGF-1. The effect of high glucose concentration of glucose on the level of p38 MAPK in BREC was examined. BREC were incubated in either 5 or 20 mmol/L glucose for 0, 15, 30, or 60 minutes. The level of activated p38 was determined by Western blotting and did not change signficantly in any of the test groups examined in 5 and 20 mmol/L glucose.

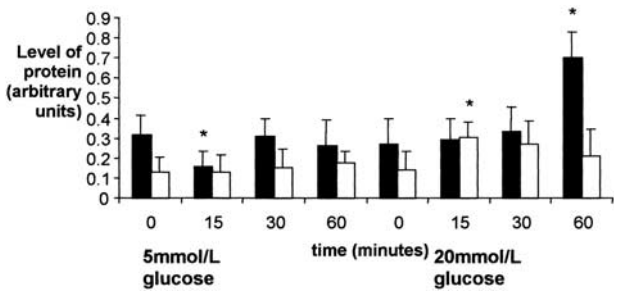

Fig 5. Effect of p38 and p42/44 MAPK activation in response to high glucose concentrations. The effect of 5 and 20 mmol/L glucose on the activation of p38 (■) and p42/44 MAPK (□) was determined by Western blotting over a 60-minute period. The results are presented as a mean of the ratio of either p38 or p42/44 MAPK divided by the total level of pERK for 3 separate experiments. When the cells were cultured in 5 mmol/L glucose, the level of activated p38 decreased at the first time point of 15 minutes ($P < .05$) and by 30 minutes and 60 minutes was relatively constant. A significant increase in elevation was noted after 60 minutes exposure to 20 mmol/L glucose ($P < .05$). The level of activated p42/44 was increased after 15 minutes exposure to 20 mmol/L ($P < .05$).

some of the dichotomy of the IGF-1 signaling regimen as seen in this study and in the reduced mitogenicity of cultured glomerular mesangial cells in high glucose.[24] Further elucidation of the mechanism of this response makes it necessary to examine in more detail the relationship between the dual properties of the regulation of cell growth and the regulation of glucose homeostasis by IGF-1 and the associated intracellular signaling cascades.

This work and that of others clearly raises questions about the role of nutrient availability and specific phosphorylation events that may lead to changes in cell behavior. The relationship that exists between glucose concentration, growth factors, and cell growth is clearly of great importance given the scope for alteration of these parameters in the development of effective preventative or therapeutic solutions to the microvascular complications of diabetes.

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