Short-term peaks in glucose promote renal fibrogenesis independently of total glucose exposure

T. S. Polhill, S. Saad, P. Poronnik, G. R. Fulcher, and C. A. Pollock

HYPERGLYCEMIA IS KNOWN TO be integral to the development of microvascular complications and a significant contributor to macrovascular complications in patients with diabetes mellitus (5-7, 16, 17). The clinical impact of hyperglycemia on total mortality (5, 6), cardiovascular (5-7, 16, 17), and cerebrovascular (17) morbidity, and less commonly for renal complications (6, 8) has been observed. Importantly, intervention studies have demonstrated that tight glycemic control reduces the risk of microvascular complications (including diabetic nephropathy and retinopathy) in both type 1 and type 2 diabetes mellitus (4, 8, 10, 23).

Many of the complications of diabetes mellitus involve a potential relationship between microvascular disease, atherosclerosis, and short-term elevations in blood glucose (9, 23). The hemoglobin A1c (HbA1c) level is strongly predictive of the development of diabetic complications (11, 23), but it is also possible that acute toxic effects of short-term hyperglycemia, not fully reflected in the HbA1c, may also be relevant. For example, within the diabetic population, postprandial plasma glucose levels independently correlate with the risk of subsequent cardiovascular disease (12), and in fact postchallenge glucose levels and glucose spikes (the difference between fasting and 2-h postchallenge glucose levels) are more predictive of macrovascular complications than either fasting plasma glucose or HbA1c measures (27). This may also be the case for microvascular disease (24).

The mechanism whereby enhanced tissue damage occurs in this setting has not been established. Prior studies assessing the cellular effects of exposure to high glucose have generally used cell culture models with continuous exposure to medium containing 25 to 30 mM glucose. There are few studies that have addressed the cellular consequences of exposure to varying concentrations of glucose. The existing studies are in a limited number of cell systems including endothelial cells (22) and mesangial cells (26) and our own previous study in tubulointerstitial cells of the kidney (15) and have demonstrated that intermittent exposure to high-glucose conditions exacerbates the deleterious effects observed in cells constantly exposed to high glucose. Significantly, these studies use prolonged exposure (i.e., 8-12 h) to 25 or 30 mM glucose. Hence, these conditions fail to mimic transient postprandial fluctuations in glucose and also did not include controls to account for the equivalent glucose load over the time period. Additionally, these studies had not been designed to isolate the specific effects of repeated short-term changes in glucose from the inherent fluctuations in osmolality.

Thus the present studies were undertaken to determine whether short-term exposure of human cortical fibroblasts (CF) to glucose concentrations that mimicked the pattern of postprandial hyperglycemia induced alterations in cell growth and matrix deposition and remodeling capacity that were independent of total glucose exposure. The specific role of fluctuations in osmolality in observed changes was additionally determined.

METHODS

Patients. Segments of macroscopically normal renal cortex were obtained under aseptic conditions from nine patients undergoing
nephrectomy for small (<6 cm) tumors. Patients were accepted for inclusion into the study if there was no history of renal or systemic disease known to be associated with tubulointerstitial pathology. In addition, the presence of a histologically normal cortical tubulointerstitium at sites removed from the tumor was confirmed by independent pathological examination. The patients were otherwise healthy and receiving no medication. Written informed consent was obtained from each patient before surgery, and ethical approval for the study was obtained from the Royal North Shore Hospital Human Research Ethics Committee.

Cell culture. The methods for primary culture of renal CF are described in detail elsewhere (14). In brief, CF were derived from the renal cortex by collagenase digestion and the resultant digest was resuspended in 45% Percoll (Amersham Pharmacia Biosciences UK, Buckinghamshire, UK) in saline and separated into four distinct bands by isopycnic ultracentrifugation. The top band was removed for renal CF culture and resuspended in DMEM/F-12 medium containing 10% fetal calf serum (Trace Biochemicals, Sydney, Australia). The cells were plated into tissue culture flasks and incubated in humidified 95% air-5% CO₂ at 37°C and the media were changed every 48 h until the cells reached confluence. The cells were then harvested using trypsin (Integrated Sciences, Sydney, Australia) and stored in liquid nitrogen. When required, the cells were thawed and after reaching confluence were subcultured into the experimental chambers.

Experimental protocol. Experiments were performed on confluent monolayers of CF. Cells were cultured for 72 h in serum-free medium containing four different “glucose conditions.” An additional osmotic control was included using the stereoisomer L-glucose, which is not transported into cells, to determine whether fluctuating osmolarity may account for any effects observed in cells exposed to fluctuating D-glucose. Experiments were performed at the conclusion of the 72-h experimental protocol: 1) control (constant exposure to 5 mM D-glucose); 2) high (constant exposure to 15 mM D-glucose); and 3) fluctuating glucose, where cells were exposed for 2 h to medium containing 15 mM D-glucose followed by 3-h exposure to 5 mM glucose medium, with this cycle repeated three times a day. Cells were then incubated for 12 h overnight in 5 mM D-glucose. This was repeated over the 72-h experimental period. In this fluctuating protocol, the cells were exposed to a total of 180 mM glucose over a 24-h period compared with 120 mM under control conditions, 4) Moderately elevated glucose involved constant exposure to 7.5 mM D-glucose. This condition gave the same total glucose load over 24 h as the fluctuating condition (180 mM) but delivered it in a constant manner. 5) In the experiments designed to differentiate the specific effect of D-glucose from fluctuations in osmotic concentrations per se, cells were exposed for 2 h to medium containing 5 mM D-glucose + 10 mM L-glucose, followed by 3-h exposure to 5 mM glucose medium, with this cycle repeated three times. These experiments are referred to subsequently as the “fluctuating osmotic control.”

All media were changed simultaneously for all conditions, and media were preequilibrated and held at 37°C in the tissue culture incubator for the duration of the experiment.

Assessment of cell growth and protein content. Total cell number was used as an indicator of cellular proliferation. Manual cell counts were performed in triplicate on trypsinized cells using a hemocytometer (LKB Wallac, Turku, Finland). The data were standardized on a per cell basis.

Collagen IV, fibronectin, and tissue inhibitor of metalloproteinases-1. Western blot analysis for tissue inhibitor of metalloproteinases (TIMP)-1, collagen IV, and fibronectin protein expression was performed. In brief, electrophoresis was performed on culture supernatants for TIMP-1 and on cell lysates for collagen IV and fibronectin Western blotting. Equal amounts of protein were loaded and all samples were subjected to SDS-PAGE under nonreducing conditions for TIMP-1 and collagen IV and reducing conditions for fibronectin. Proteins were then transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biosciences UK). Nonspecific binding sites were blocked overnight (5% nonfat milk and 0.1% Tween 20 in PBS) after which the membranes were incubated overnight in a 1:100 dilution of mouse monoclonal anti-fibronectin antibody (Neomarkers), or for 2 h in a 1:300 dilution of mouse monoclonal anti-TIMP-1 antibody (Oncogene, San Diego, CA), or for 2 h in a 1:5,000 dilution of rabbit monoclonal anti-collagen antibody (Abcam, Cambridge, UK), followed by washing four times after which they were incubated with peroxidase-labeled secondary antibodies (Amersham Pharmacia Biotech) for 1 h and again washed four times. The blots were then detected using ECL (Amersham Pharmacia Biotech). The bands corresponding to fibronectin and TIMP-1 were quantitated using National Institutes of Health Image software version 1.60.

Matrix metalloproteinase-2 and -9 secretion. Gelatin zymography was used to determine the levels of the gelatinases matrix metalloproteinase (MMP)-2 and MMP-9 in the CF culture supernatant. Culture supernatants were harvested after the final 12 h of the 72-h protocol, centrifuged at 12,500 g for 5 min, mixed with gel sample buffer, and separated electrophoretically on 10% nonreducing SDS-polyacrylamide gels containing 1 mg/ml gelatin. The gels were then incubated for 1 h at room temperature in 2.5% Triton X-100. The gels were rinsed and incubated overnight at 37°C in buffer (50 mmol/l Tris-HCl, 100 mmol/l NaCl, 10 mmol/l CaCl₂, 0.02% NaN₃, pH 7.5) and counterstained for 1 h in 0.2% Coomassie blue R-250 (Bio-Rad) dissolved in 50% ethanol/10% acetic acid. The lytic bands representing MMP activities were quantified using standard densitometric techniques and standardized to cell number.

Real-time RT-PCR. Real-time RT-PCR was performed for the quantification of transforming growth factor (TGF)-β mRNA induced by exposure to 7.5 mM D-glucose, short-term exposure to 15 mM L-glucose, and sustained or short-term exposure to 15 mM D-glucose compared with 5 mM D-glucose. RNA was extracted using an RNaseasy Mini kit (Qiagen, Victoria, Australia) as per the manufacturer’s instructions after 90-min, 24- and 48-h exposure to 7.5, 7.5, and 15 mM glucose. The following sequence-specific primers and probes for human TGF-β were designed: forward, 5'-GCAACAT-GTTGAACTCTACCAGAA-3'; reverse 5'-GACGTCAAAAGA-GCTGACCC-TAMRA-3' and probe 5'-FAM-ACCTTGGTAACCGGCT-GCTGACCC-TAMRA-3' (31) using a TaqMan One-Step RT-PCR Master Mix Reagents Kit (PE Applied Biosystems, Foster City, CA). The forward and reverse sequences of the primers were designed for different exons of the gene to eliminate detection of genomic DNA. For precise quantitative analysis of gene expression, the predeveloped TaqMan Assay Reagents (Endogenous Control Ribosomal RNA Control [18S rRNA]) (PE Applied Biosystems) were included in the RT-PCR reactions. All samples were assayed in triplicate, presented as fold-increase compared with time 0. Data from the reaction were collected and analyzed by the complementary computer software (28).

Statistical analysis. Results are expressed as means ± SE, as a percentage of the control (cells exposed continuously to 5 mM glucose) value. Individual experiments between three and nine different patients were included in each experimental group, and each experiment was performed in triplicate. Statistical comparisons between groups were made by ANOVA, with pairwise comparisons made by paired t-test. Analyses were performed using the software package Statview version 4.5 (Abacus Concepts, Berkeley, CA). P values <0.05 were considered significant.
RESULTS

Cell growth parameters. Exposure of CF to each of the experimental conditions over a 72-h period had no significant effect on cellular proliferation ($n = 9$; data not shown). Similarly, exposure to the different d-glucose concentrations and the fluctuating osmotic control had no effect on total cellular protein content ($n = 9$; data not shown).

[^H]proline incorporation. Exposure to either 15 or 7.5 mM glucose for 72 h had no significant effect on the levels of tritiated proline incorporation, being 96.7 ± 9.7 and 94.8 ± 5.3% of control values, respectively. However, in CF exposed to fluctuating d-glucose, there was a significant increase in proline incorporation to 114.3 ± 7.2% compared with control ($P < 0.05$) and cells exposed to the same overall d-glucose load ($P < 0.05$; Fig. 1). In cells exposed to the fluctuating osmotic control, proline synthesis was also increased to 121.9 ± 6.8% compared with control ($P < 0.01$). This increase was not statistically different to that observed in cells exposed to the fluctuating d-glucose protocol (Fig. 1).

Collagen IV production. Exposure of CF to either 7.5 mM d-glucose or 15 mM d-glucose for 72 h had no significant effect on the levels of collagen IV production, being 106.7 ± 13.8% ($P = 0.05$ vs. control) and 123 ± 12.4% ($P = 0.05$) of control values, respectively. However, in CF exposed to fluctuating d-glucose, there was a significant increase in collagen IV production to 153.9 ± 12.5% of control values ($P < 0.005$). Cells exposed to the fluctuating osmotic control also had an increase in collagen IV production (112.5 ± 1.2%; $P < 0.05$ vs. control). However, the increase was significantly less than that observed in the cells exposed to fluctuating d-glucose ($P < 0.01$). Representative Western blot analysis and quantitative expression are demonstrated in Fig. 2, A and B, respectively. The results from both the[^H]proline incorporation and the collagen IV Western blot analysis indicate that an increase in collagen production occurred only in cells exposed to fluctuating d-glucose and the effect was only, in part, due to osmotic-dependent mechanisms.

Fibronectin secretion. Exposure of CF to 7.5 mM d-glucose for 72 h had no significant effect on fibronectin secretion, being 105.7 ± 21.4% ($P = 0.05$) of control. However, exposure to 15 mM or fluctuating d-glucose for 72 h induced similar increases in fibronectin secretion, being 176.6 ± 9.4% ($P < 0.001$) and 183.0 ± 17.5% ($P < 0.001$) of control values, respectively. Exposure to the fluctuating osmotic control increased fibronectin to 134.8 ± 12.8% ($P < 0.05$ vs. control) but was significantly lower than observed in the cells exposed to fluctuating d-glucose. Representative Western blot analysis and quantitative expression are demonstrated in Fig. 3, A and B, respectively.

MMP-9 and MMP-2 secretion. Fluctuating d-glucose, exposure to 7.5 mM d-glucose, and exposure to the fluctuating osmotic control had no significant effect on the activity of MMP-9 (Fig. 4A). Similarly, there was no significant effect on the levels of MMP-2 under these conditions (Fig. 4B). In contrast, constant exposure to 15 mM d-glucose resulted in significant increases in the activities of both MMP-9 (115 ± 6%; $P < 0.05$) and MMP-2 (118 ± 8%; $P = 0.01$; Fig. 4, A and B). These data show that under fluctuating d-glucose and in the fluctuating osmotic control, there is no increase in matrix-degrading enzymes to parallel the increase in collagen synthesis. The increases in MMP activity observed during sustained 15 mM d-glucose exposure in the absence of an increase in collagen synthesis are suggestive of enhanced matrix-remodeling capacity under these conditions.

TIMP-1 expression. Exposure of CF to constant levels of 15 or 7.5 mM d-glucose had no significant effect on the levels of TIMP-1. However, in cells exposed to fluctuating glucose, there was a significant increase ($P < 0.05$) compared with all other experimental conditions to 139 ± 10% of control levels (Fig. 5). These data reinforce the potentially deleterious effects of fluctuating d-glucose in that an increase in TIMP-1 would

Fig. 1. Cellular proline incorporation under the defined experimental conditions. Results are means ± SE and expressed as a percentage of 5 mM glucose (control). Fluct., fluctuating. *$P < 0.05$ vs. control. **$P < 0.01$ vs. control. #$P < 0.05$ vs. 7.5 mM glucose ($n = 8$).

Fig. 2. A: representative Western blot analysis demonstrating increased collagen IV protein in cells exposed to fluctuating glucose conditions, but no increase in cells exposed to 15 or 7.5 mM glucose. B: quantitative representation of collagen IV Western blot analysis. Results are means ± SE and expressed as a percentage of 5 mM glucose. *$P < 0.05$ vs. control. **$P < 0.005$ vs. control. #P < 0.001 vs. fluctuating d-glucose ($n = 5$).
further serve to inhibit matrix degradation and potentiate the effects of increased collagen synthesis, as reflected by the increased proline incorporation and Western blot analysis for collagen IV. This situation does not arise in CF exposed to a similar total glucose load delivered over the same time period, or during a sustained exposure to high-glucose conditions. As no effect was observed in cells exposed to the fluctuating osmotic control, the induction of TIMP-1 by fluctuating D-glucose is unlikely to be due to osmotic changes.

**TGF-β1 mRNA expression.** In keeping with the profibrotic environment generated by the effects of fluctuating D-glucose on collagen synthesis, the transition from 5 to 15 mM D-glucose induced a 2.2 ± 0.4-fold increase in the expression of TGF-β1 mRNA within 90 min compared with control levels (P < 0.05). A similar increase was observed in cells exposed to the fluctuating osmotic control at 90 min (1.8 ± 0.1-fold increase compared with control; P < 0.05). No increase in TGF-β1 mRNA expression was observed in cells exposed to 7.5 mM glucose within 90 min. However, 24-h exposure to both 7.5 and 15 mM D-glucose and the osmotic control induced an increase in TGF-β1 mRNA expression (1.87 ± 0.1, P < 0.05; 2.5 ± 0.4, P < 0.005; and 2.47 ± 0.4, P < 0.005), which normalized in both experimental conditions to control values by 48 h (Fig. 6).

**DISCUSSION**

The results of these experiments clearly demonstrate the deleterious effects of intermittent elevations in glucose concentrations on the fibrotic process that occurs in the human kidney. Specifically, the CF exposed to fluctuating glucose levels demonstrate an increase in both collagen and noncollagen matrix production and a reduction in MMP activity compared with cells exposed to a similar overall glucose load and indeed to cells exposed to a sustained elevation in glucose concentration. The findings suggest that excessive extracellular matrix accumulation will occur in the renal tubulointerstitium.

**Fig. 3.** A: representative Western blot analysis demonstrating increased fibronectin protein in cells exposed to constant 15 mM glucose and fluctuating glucose conditions, but no increase in cells exposed to 7.5 mM glucose. B: quantitative representation of fibronectin Western blot analysis. Results are means ± SE and expressed as a percentage of 5 mM glucose. *P < 0.05 vs. control. **P < 0.001 vs. control. #P < 0.05 vs. fluctuating D-glucose (n = 7).

**Fig. 4.** Matrix metalloproteinase (MMP)-9 (A) and MMP-2 (B) secretion from cells exposed to the defined experimental conditions. Results are expressed as a percentage of 5 mM glucose (control). *P < 0.05 vs. control. **P < 0.01 vs. control (n = 5).

**Fig. 5.** Tissue inhibitor of metalloproteinases (TIMP)-1 secretion from cells exposed to the defined experimental conditions. Results are expressed as a percentage of 5 mM glucose (control). *P < 0.05 vs. all other experimental conditions (n = 6).
GLUCOSE PEAKS INDUCE RENAL FIBROGENESIS

The direct effect of fluctuating glucose on tissue pathology in a manner that mimics postprandial excursions in glycemic control has not been previously studied. We previously showed that changing media glucose concentrations every 24 h results in enhanced tubulointerstitial pathology (15), and similar deleterious responses have been observed in mesangial cells (26) and endothelial cells (22). The present study showed that more frequent changes in glucose may amplify the deleterious effects observed. Specifically, exposure to high glucose may acutely alter intracellular calcium (25) and cell signaling pathways (1, 20) and induce oxidative stress (2, 3, 20) and short-term changes in the expression of mRNA for deleterious cytokines (21). Our studies demonstrated that TGF-β1 is induced acutely by the transition from 5 to 15 mM glucose. Although upregulation of mRNA persists for 24 h in cells constantly exposed to either 7.5 or 15 mM glucose, it has reverted to baseline by 48 h. Hence, repeated activation of potentially deleterious responses, such as TGF-β1, may induce more cellular and hence organ pathology than when glucose levels are high but stable.

Prior studies demonstrated a profibrotic effect in fibroblasts following exposure to glucose concentrations of greater than 25 mM glucose (18). Clearly, the clinical relevance of these experiments is likely to be minimal as most patients do not have prolonged periods with blood glucose levels that are persistently at this level. However, there is limited evidence to suggest that exposure to glycemic levels lower than this in the kidney is associated with oxidative stress (19) and tissue pathology. Indeed, the present study suggests that in contrast to the more consistent profibrotic effects that occur in fibroblasts exposed to 25 mM glucose, the effects of constant exposure to 15 mM glucose are limited to an increase in fibronectin and an increase in TGF-β1 that normalizes within 48 h.

The role of changes in osmolality in inducing the observed profibrotic changes was additionally assessed in the present study. Clearly, TGF-β1 mRNA was similarly induced by short-term exposure to D-glucose and L-glucose. Fluctuating L-glucose also induced an increase in both collagen and fibronectin, however, to a lesser extent than observed with D-glucose. In keeping with these results, the effects of glucose on cell cytokine production have previously been demonstrated to be due to both osmotic- and nonosmotic-dependent mechanisms (30). Specifically, in mesothelial cells increased profibrotic and proinflammatory cytokines following exposure to high glucose are, in part, induced by increased osmolality and, in part, induced by activation of the polypeptide pathway (30). One of the most significant consequences of fluctuating osmolality, inherent in exposure to high D-glucose, is a fluctuation in cell volume. It has been previously recognized that TGF-β1 up-regulates the transcription of the serum- and glucocorticoid-dependent kinase hSGK1, which is known to be important in normalization of cell volume through its effects on key transport proteins (29). This kinase has also been demonstrated to stimulate cell growth and proliferation through a variety of growth factors (13), which may contribute ultimately to enhanced matrix production (13). Hence, osmotically and nonosmotically induced changes in cellular function are likely to contribute to the pathology observed.

In summary, this study provides evidence that short-term repeated exposure to high-glucose conditions amplifies the adverse cellular responses compared with cells exposed to the same glucose load or constantly high levels of glucose. These effects are shown to be mediated through both osmotically and nonosmotically induced mechanisms. These results are likely to have important implications for the monitoring and treatment strategies implemented in patients with diabetes mellitus.
ACKNOWLEDGMENTS

We acknowledge the staff at the Department of Physiology at the University of Sydney for providing the facilities to set up the real-time RT-PCR experiments.

GRANTS

This work was partly supported by a grant provided by the Juvenile Diabetes Research Foundation and The National Health and Medical Research Council and by an unrestricted research grant from Novartis Pharmaceuticals Australia.

REFERENCES