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. Short-term peaks in glucose promote renal fibrogenesis independently of total glucose exposure. Am J Physiol Renal Physiol 287: F268–F273, 2004. First published April 27, 2004; 10.1152/ajprenal.00084.2004.—Postprandial hyperglycemia is implicated as a risk factor predisposing to vascular complications. This study was designed to assess recurrent short-term increases in glucose on markers of renal fibrogenesis. Human renal cortical fibroblasts were exposed to fluctuating short-term (2 h) increases to 15 mM d-glucose, three times a day over 72 h, on a background of 5 mM d-glucose. To determine whether observed changes were due to fluctuating osmolality, identical experiments were undertaken with cells exposed to L-glucose. Parallel experiments were performed in cells exposed to 5 mM d-glucose and constant exposure to either 15 or 7.5 mM d-glucose. Fluctuating d-glucose increased extracellular matrix, as measured by proline incorporation (P < 0.05), collagen IV (P < 0.005), and fibronectin production (P < 0.001), in association with increased tissue inhibitor of matrix metalloproteinase (MMP) (P < 0.05). Sustained exposure to 15 mM d-glucose increased fibronectin (P < 0.001), in association with increased MMP-2 (P = 0.01) and MMP-9 activity (P < 0.05), suggestive of a protective effect on collagen matrix accumulation. Transforming growth factor-β1 (TGFB1) mRNA was increased after short-term (90 min) exposure to 15 mM glucose (P < 0.05) and after 24-h exposure to 7.5 mM d-glucose (P < 0.05). Normalization of TGFB1 secretion occurred within 48 h of constant exposure to an elevated glucose. Fluctuating l-glucose also induced TGFB1 mRNA and a profibrotic profile, however, to a lesser extent than observed with exposure to fluctuating d-glucose. The results suggest that exposure to fluctuating glucose concentrations increases renal interstitial fibrosis compared with stable elevations in d-glucose. The effects are, in part, due to the inherent osmotic changes.

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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, athero-

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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. The total protein content of cells was determined as a marker of cell proliferation. Manual cell counts were performed in triplicate on trypsinized cells using a hemocytometer (LKB Wallac, Turku, Finland). The data were standardized as fold-increase compared with 5 mM D-glucose.

**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 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 quantitated 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)-β1 mRNA induced by exposure to 7.5 mM D-glucose, short-term exposure to 15 mM D-glucose, and sustained or short-term exposure to 15 mM D-glucose compared with 5 mM D-glucose. RNA was extracted using an RNeasy Mini kit (Qiagen, Victoria, Australia) as per the manufacturer’s instructions after 90-min, 24- and 48-h exposure to 5, 7.5, and 15 mM glucose. The following sequence-specific primers and probes for human TGF-β1, which were designed for TGF-β1 forward, 5’-GCACCACCATGTGGAACTCTACCTCAAGA-3’; reverse 5’-GACGTCAAAAGATCAGCCCACTCA-3’ and probe 5’-FAM-ACCTTGTTGACCCGGTGTCGTTACCCC-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.

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RESULTS

Cell growth parameters. Exposure of CF to either 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).

[^3]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% 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 = not significant (NS)] and 123 ± 12.4% (P = NS) 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 [^3]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 = NS) 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. 4A 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...
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 non-collagen 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).
of patients exposed to significant glycemic variability. The results additionally suggest that at least part of the deleterious effects of high glucose is due to inherent changes in osmolality. As tubulointerstitial fibrosis is the hallmark of progressive diabetic nephropathy, these findings are consistent with the clinical observation that postprandial elevations in blood glucose are associated with an increased risk of renal disease in patients with diabetes mellitus (24).

The results suggest that the adverse effects of short-term increases in blood sugar are independent of the total glucose exposure. Constant exposure to a moderate elevation in glucose, which results in equivalent glucose “load,” did not have any deleterious effect on the CF growth or matrix accumulation and degradation. Indeed, in several parameters exposure of the cells to constantly high glucose levels, which doubled the glucose exposure to 360 mM glucose per day, induced less pathology compared with those exposed to fluctuating levels of glucose. Specifically, when glucose exposure was constant there was no increase in collagen production, and an increase in MMP-2 and MMP-9 activity and no change in TIMP-1 expression, thus mitigating against matrix accumulation. Exposure to fluctuating glucose induced no change in MMP-2 or MMP-9 and increased TIMP-1 expression, suggesting that the net effect of fluctuating glucose levels is more likely to predispose to renal pathology. These results support the recommendation for postprandial monitoring of glucose levels in patients with diabetes mellitus and imply that important differences in end organ damage could occur in individuals with similar HbA1c but different postprandial glucose levels. Although acute hyperglycemia has been considered to exert deleterious effects on tissue through mechanisms that may partly involve nonenzymatic glycation (3), the results of the present study would not support this as being the main cause of accelerated organ pathology as similar degrees of protein glycation would be likely to occur following exposure to similar overall glucose loads.

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 each 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.
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