Stimulation of Urinary TGF-β and Isoprostanes in Response to Hyperglycemia in Humans

Tracy A. McGowan,* Stephen R. Dunn,† Bonita Falkner,‡ and Kumar Sharma*†

*Center for Diabetic Kidney Disease, †Cell and Molecular Biology of Kidney Disease-Dorrance Hamilton Research Laboratories, and ‡Center for Hypertension, Division of Nephrology, Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania

TGF-β and oxidant stress have been considered to play key roles in the pathogenesis of diabetic vascular complications; however, the stimulus for these factors in humans is not clear. The purpose of this in vivo study was to determine whether transient hyperglycemia in humans is sufficient to increase renal production of TGF-β1 and urinary isoprostanes in normal humans. A hyperglycemic clamp procedure was performed on 13 healthy volunteers. An infusion of glucose was delivered to maintain the plasma glucose between 200 and 250 mg/dl for 120 min. Timed urine samples, collected on an overnight period before the study, at each void on completion of the procedure, and the following overnight, were assayed for TGF-β1, F2-isoprostanes, and creatinine. Plasma samples were assayed for TGF-β1 before and at timed intervals throughout hyperglycemia. Mean baseline TGF-β1 in plasma was 4.57 ± 0.22 ng/ml, and no change in plasma TGF-β1 levels was detected throughout the hyperglycemia period. Baseline urine TGF-β1 was 4.14 ± 1.16 pg/mg creatinine. The fractional urine samples showed a sharp increase in TGF-β1 excretion in the 12-h period after exposure to hyperglycemia, with a mean peak TGF-β1 of 30.43 ± 8.05 pg/mg (P = 0.002). TGF-β1 excretion in the subsequent overnight urine sample was not different from baseline (4.62 ± 1.21 pg/mg). Urinary isoprostanes increased from a baseline of 4.92 ± 0.74 to 13.8 ± 3.37 ng/mg creatinine. It is concluded that 120 min of hyperglycemia in normal humans is sufficient to induce an increase in renal TGF-β1 and isoprostane production.


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athologic characteristics of diabetic nephropathy include early increases in glomerular and tubular compartments followed by progressive thickening of glomerular basement membranes and mesangial matrix accumulation. Clinically, these changes are associated with augmented glomerular blood flow in the early stages and enhanced glomerular permeability to albumin with a decline in GFR in the later stages (1). The mechanisms that initiate and mediate the renal pathology in patients with diabetes are not fully resolved but seem to involve regulatory alterations within the tissue. In vitro studies have shown that exposure of renal cells to high ambient glucose stimulates hypertrophy and production of collagen types I and IV in proximal tubular and mesangial cells (2–4). On the basis of experimental studies, the accumulation of excess matrix, through both increased production and decreased degradation, seems to advance the diabetic renal pathology (5). Two of the pathways that may mediate the renal complications of diabetes include TGF-β and reactive oxygen species (ROS) generation (6).

TGF-β has been found to stimulate extracellular matrix proteins including fibronectin, type IV and type I collagen. TGF-β also impairs proteolytic degradation of collagen fibers through a decrease in synthesis and secretion of matrix metalloproteinases and an increase in synthesis of tissue inhibitors of metalloproteinases (7). In vitro studies have detected an increase in TGF-β1 mRNA expression and bioactivity in mesangial and tubular cells when exposed to high glucose concentration (8). Renal TGF-β1 mRNA levels are also increased in diabetic mice and rats within 48 h after onset of hyperglycemia. Recent studies that evaluated glucose-induced TGF-β1 production demonstrated a key role for the upstream stimulatory factor family of transcription factors in vitro as well as in vivo (9,10). The causative role of TGF-β in stimulating early gene expression of matrix molecules as well as mesangial matrix accumulation is supported by experiments with neutralizing antibodies in streptozotocin-induced diabetic mice and in db/db mice (11,12). In patients with diabetes, there is increased renal production of TGF-β in both early and advanced disease (13,14). However, it remains unclear whether transient hyperglycemia itself is sufficient to stimulate renal TGF-β production in humans.

Increased oxidant stress has also been recognized to play a key role in mediating glucotoxicity in a variety of animal models of diabetic complications (15,16). Recent laboratory investigations have identified a link between oxidized free radicals and upregulation of TGF-β activity (17,18). In the streptozotocin-induced diabetic rat model, Montero et al. (19) found a marked increase in plasma levels and urinary excretion of
F₂-isoprostane. In tissue culture, a high ambient glucose increased F₂-isoprostane synthesis in glomerular endothelial and mesangial cells. Incubation of glomerular cells with F₂-isoprostanes stimulated the production of TGF-β (18). It is interesting that TGF-β has also been recognized to stimulate ROS production in renal and vascular cells, suggesting that there may be a positive feedback pathway between TGF-β and ROS (20).

In patients with established diabetes, there are numerous acute and chronic metabolic derangements as well as hemodynamic stresses that all may contribute to increased production of renal TGF-β and increase oxidant stress. These factors include hyperglycemia, hexosamines, glycated proteins, protein kinase C activation, and increased glomerular pressure. Although many pathways are stimulated within hours of exposure to hyperglycemia, some pathways require weeks to months to occur, e.g., glycation of proteins. Therefore, it is difficult to identify the key components that may provide the dominant stimulus for renal TGF-β production in humans with diabetes. In an attempt to limit the number of variables involved, we performed a study to raise glycermia levels in normal volunteers and measured levels of TGF-β and isoprostanes.

**Materials and Methods**

**Human Protocol**

Healthy volunteers were enrolled in this study after written informed consent was obtained on a Thomas Jefferson University approved Institutional Review Board protocol. Each volunteer was instructed to obtain a timed overnight urine sample and report to the Renal Clinical Research Unit at 8 a.m. after a 12-h overnight fast. The overnight urine sample served as the baseline sample for urine TGF-β and F₂-isoprostanes. Venous catheters were placed in each arm, one for infusion and one for obtaining blood samples. A fasting blood sample was obtained for measurement of baseline plasma glucose and TGF-β. An infusion of 20% glucose was delivered in bolus to raise the plasma glucose to 200 mg/dl. Plasma glucose was measured every 5 min, and the glucose infusion rate then was adjusted to maintain the plasma glucose between 200 and 250 mg/dl for 120 min. The plasma glucose then was tapered to 120 mg/dl by reduction in the glucose infusion rate over the next 30 min. Urine samples were collected at the end of the infusion and on each void thereafter during the day of the infusion. Another timed overnight urine sample was collected the next morning. The urine samples were assayed for urine creatinine and TGF-β. Plasma samples were collected before the start of the infusion and at 60-min intervals throughout the hyperglycemic infusion and assayed for TGF-β and insulin. To determine whether there was evidence of oxidative stress from the steady-state hyperglycemia, F₂-isoprostanes were also measured in the urine samples. Glucose was analyzed by the glucose oxidase technique with the Glucostat analyzer (Model 27; YSI Inc, Yellow Springs, OH). Creatinine concentration in urine and plasma samples was assayed using a NOVA analyzer.

**TGF-β Measurements**

TGF-β in urine was assayed by a method previously described and developed by Sharma and Dunn (www.jeffersonhospital.org/cdkd) (21). This assay uses a sandwich ELISA (Quantikine kit for Human TGF-β1 Immunoassay; R&D Systems, Minneapolis, MN). Urine samples were initially concentrated and pH was adjusted to activate latent TGF-β to active TGF-β. Corrections were made for urine concentration by measuring urinary creatinine, and values were expressed as TGF-β1 in pg/g creatinine. The correlation coefficient with standards is >0.98, and the lowest detectable limit for measurement is 0.7 pg/ml. The reliability of this assay for urinary TGF-β1 is high with an intra-assay coefficient of variation of 2.5 ± 3.0% and an interassay coefficient of variation of 5.6 ± 4.2%. Recovery of fortified TGF-β1 added to urine samples was 94%. No interference from TGF-β2 was observed. Plasma levels of TGF-β1 were measured using the method of Wakefield et al. (22).

**Measurement of Urinary F₂-Isoprostanes**

Isoprostanes are prostaglandin-like compounds that are produced by free radical–mediated peroxidation of lipoproteins. Urinary levels of 15-isoprostane F₂α (also known as 8-epi-PGF₂α, or 8-iso-PGF₂α) has been used for the noninvasive assessment of oxidative stress in patients with diabetes and experimental models of diabetes (19,23). It has been shown that unmetabolized cyclo- oxygenase–derived prostaglandins in urine derive almost exclusively from their local formation in the kidney. In this study, we measured urinary levels of 15-isoprostane F₂α using a competitive ELISA (Oxford Biomedical Research, Oxford, MI). In this assay, urine samples are mixed with an enhancing reagent that essentially eliminates interferences as a result of nonspecific binding. The 15-isoprostane F₂α in the samples competes with 15-isoprostane F₂α conjugated to horseradish peroxidase for binding to a polyclonal antibody specific for 15-isoprostane F₂α coated on the microplate. The horseradish peroxidase activity results in color development when substrate is added, with the intensity of the color proportional to the amount of 15-isoprostane F₂α bound and inversely proportional to the amount of unconjugated 15-isoprostane F₂α in the samples. A Spectrax max 250 plate reader (Molecular Devices, Sunnyvale, CA) was used to measure the absorbance at 450 nm. Samples were run with known standards. Urinary samples were also run in duplicate, and the average of these values was used to determine the corresponding 15-isoprostane F₂α concentration from the standard curve. The manufacturer’s reported correlation of this immunoassay when compared with gas chromatography/mass spectrometry of the same human urine samples is >0.8. Use of this assay in our laboratory indicates reliable results with a low coefficient of variation (<5%).

**Statistical Analyses**

Unless otherwise stated, arithmetic means and SEM are reported. A paired t test was used to compare baseline levels with peak levels during and after the glycemic clamp. For comparison of three variables (baseline, after glucose clamp, and after overnight), a single-factor ANOVA with repeated measure was used. P < 0.05 for a two-sided test was considered significant.

**Results**

Complete data were obtained on 13 volunteers. The clinical characteristics are reported in Table 1. The sample included six men and seven women with a mean age of 39 yr. The ethnic distribution of the sample was 10 white, two Asian, and one Hispanic. All volunteers had BP in the normal range and no evidence of renal dysfunction.

Before the hyperglycemic clamp, all patients had levels of fasting blood glucose in the normal range (Table 2). During the hyperglycemic clamp, the blood glucose ranged between 200 and 280 mg/dl beginning at approximately 15 min after the beginning of glucose infusion and lasting for 120 min. There was an expected rise in plasma insulin levels during the hyperglycemic clamp. The mean baseline TGF-β1 level in plasma was
4.57 ± 0.22 ng/ml. No change in plasma TGF-β levels was detected during the hyperglycemia period (Table 2).

The mean baseline urine TGF-β1 level (corrected for creatinine excretion) was 4.14 ± 1.16 pg/mg creatinine. Figure 1 depicts the urinary TGF-β1 excretion before and after the 2-h period of steady-state stable hyperglycemia. The fractional urine samples showed a sharp increase (seven-fold) in TGF-β1 excretion (30.43 ± 8.05 pg/mg; \( P = 0.0002 \)) within the 12-h period after exposure to hyperglycemia. The time from the beginning of the glucose infusion to a mean peak TGF-β1 excretion was 7.44 ± 1.36 h. TGF-β1 excretion in the subsequent overnight urine sample after the hyperglycemic clamp was not significantly different from baseline (4.6 ± 1.21 pg/mg; \( P = 0.760 \)).

To determine whether evidence of oxidative stress could be detected in response to hyperglycemia, we assayed F₂-isoprostane on the urine samples that were collected before and after the glucose infusion. The results of the assays on F₂-isoprostane excretion are depicted in Figure 2. The mean baseline F₂-isoprostane excretion, on the overnight samples before glucose infusion, was 4.92 ± 0.74 ng/mg creatinine. After the glucose infusion, F₂-isoprostane excretion increased to 13.81 ± 3.37 ng/mg creatinine (\( P = 0.001 \)). The mean level reverted back to baseline in the next overnight sample (5.06 ± 0.67 ng/mg creatinine; \( P = 0.882 \)).

**Discussion**

These data indicate that exposure to hyperglycemia in the range of 200 to 250 mg/dl for a period of 2 h is sufficient to upregulate urinary TGF-β1 and isoprostane excretion in healthy nondiabetic volunteers. It is of note that this level of hyperglycemia approximates the glycemic load observed in suboptimal control of patients with diabetes. In addition, patients with established diabetes will often have transient levels of hyperglycemia that exceed 200 mg/dl. The upregulation of TGF-β1 identified in the urine could not be detected in the plasma, suggesting that TGF-β1 is produced primarily in renal tissue.

Our data are supportive of previous studies that showed elevations in urinary levels of TGF-β in patients who have diabetes without a corresponding elevation in systemic levels of TGF-β1 (13,24). Urinary levels of TGF-β are considered to be a valid measure of renal production of TGF-β1, as in our previous study we found that net renal production of TGF-β1 was associated with increased urine levels (13).

Our data demonstrate that hyperglycemia by itself is a potent stimulus for renal TGF-β and is likely relevant to the human diabetic condition. The relatively modest levels of hyperglycemia induced in this study are commonly seen in patients with diabetes, especially after exposure to glycemic loads. The short duration of hyperglycemia is insufficient for advanced glyca-
tion of proteins by either Amadori modification or advanced glycation; thus, our study also determines that short-term hyperglycemia-induced stimulation of renal TGF-β1 production likely is not dependent on glycated proteins. Other pathways that are considered to mediate glucotoxicity, such as the hexosamine pathway, protein kinase C activation, extracellular signal–regulated kinase activation, and hemodynamic stress, may be involved in mediating this acute effect of hyperglycemia on renal TGF-β production (25,26). However, several cell culture studies suggest that activation of these pathways requires 24 h or more of sustained high glucose (27,28). An effect of modest hyperglycemia on the renin-angiotensin system is another potential pathway that may mediate upregulation of renal TGF-β1 production (29,30). It is likely that more than one pathway may be activated within hours of transient hyperglycemia as numerous hemodynamic and cellular systems are activated by glucose stress. Data from this study indicate that even short-term hyperglycemia (120 min) is sufficient to mediate glucotoxic pathways that culminate in production of TGF-β1 in the human kidney. Of note, the marked increase in urinary TGF-β1 protein occurred much quicker and with greater magnitude than has been found in studies with cell culture (31). It is likely that multiple pathways are stimulated by transient glucose elevations in vivo and are unable to be mimicked adequately in a cell culture system.

There was no observed increase in plasma TGF-β1 levels during the 120 min of hyperglycemia. It is possible that there could have been a delayed response in the postinfusion period that coincided with the urine peak. A limitation of this study was that plasma TGF-β1 levels after hyperglycemia were not available.

A role for insulin to raise TGF-β1 is possible as insulin levels rise dramatically with the glycemic infusion. However, we have not found evidence of increased urine levels of TGF-β in a separate study wherein patients underwent euglycemic hyperinsulinemic clamps (unpublished data). A role for osmotic stress may have contributed to the increase in urinary TGF-β1. Although cell culture studies with osmotic controls for n-glucose do not elicit TGF-β1 production (31), an osmotic control was not included and remains a limitation of our study.

It was demonstrated that high glucose stimulates free radicals (32,33), and this may be the link between hyperglycemia and the subsequent glucotoxic pathways that lead to complications of diabetes (34). In fact, studies by Brownlee and colleagues (17,33) have found that high glucose stimulation of TGF-β1 and cyclo-oxygenase 2 in cell culture may be mediated by ROS generation via the mitochondria. Our studies are supportive of this concept as urinary F2-isoprostane levels increased after short-term hyperglycemia, which would be consistent with ROS generation in the kidney. Whether ROS generation is upstream and/or downstream of renal TGF-β production remains to be determined. The source of oxidant stress with transient hyperglycemia is unclear but likely reflects renal and possibly systemic oxidant production.

Recent studies have suggested that the prediabetic condition of impaired glucose tolerance is associated with an increase in cardiovascular events as well as kidney disease (35,36). Our data are supportive of the concept that even modest levels of hyperglycemia are sufficient to impose glucotoxic effects on renal cells as evidenced by urinary F2-isoprostane and TGF-β1 levels. This is an important finding in light of the data presented by Ceriello et al. (37) that individuals with diabetes have an increased level of free radical generation after a standard meal compared with individuals without diabetes. Coutinho et al. (38) also reported that there is a positive correlation between glucose levels and cardiovascular risk that extends even below the diabetic threshold.

**Conclusion**

We have found that short-term modest hyperglycemia is a potent stimulus of renal TGF-β1 production and oxidative stress generation in normal humans. The further study of these
two mediators in relation to candidate stimuli in patients with diabetes will lead to a better understanding of the complex relationships between glucose homeostasis, TGF-β1, and the oxidative stress pathways. A better understanding of these relationships will hopefully lead to targeted therapeutic paradigms to alter disease course.

Acknowledgments
The studies were partially funded by grants to B.F. (NIH DK046107 and HL51547) and K.S. (NIH DK63017).

Portions of this article were presented in abstract form at the American Society of Nephrology Meeting; San Francisco, CA, October 10 to 17, 2001.

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