Prevention of Incipient Diabetic Nephropathy by High-Dose Thiamine and Benfotiamine

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Accumulation of triosephosphates arising from high cytosolic glucose concentrations in hyperglycemia is the trigger for biochemical dysfunction leading to the development of diabetic nephropathy—a common complication of diabetes associated with a high risk of cardiovascular disease and mortality. Here we report that stimulation of the reductive pentosephosphate pathway by high-dose therapy with thiamine and the thiamine monophosphate derivative benfotiamine countered the accumulation of triosephosphates in experimental diabetes and inhibited the development of incipient nephropathy. High-dose thiamine and benfotiamine therapy increased transketolase expression in renal glomeruli, increased the conversion of triosephosphates to ribose-5-phosphate, and strongly inhibited the development of microalbuminuria. This was associated with decreased activation of protein kinase C and decreased protein glycation and oxidative stress—three major pathways of biochemical dysfunction in hyperglycemia. Benfotiamine also inhibited diabetes-induced hyperfiltration. This was achieved without change in elevated plasma glucose concentration and glycated hemoglobin in the diabetic state. High-dose thiamine and benfotiamine therapy is a potential novel strategy for the prevention of clinical diabetic nephropathy.
***Diabetes* 52:2110–2120, 2003**

Nephropathy is a common complication of diabetes. It is characterized by the development of proteinuria, culminating in end-stage renal disease with a particular high risk of cardiovascular morbidity and mortality (1). The initial stage of development of nephropathy, incipient nephropathy, is characterized by the onset of persistent microalbuminuria and hyperfiltration. Hyperglycemia is a risk factor for the development of incipient nephropathy in both type 1 and type 2 diabetic subjects (2–4). Tight control of blood glucose (and blood pressure) decreases the risk of developing nephropathy but is not always achievable because of limitations of current drug therapy (5).

High plasma glucose concentration leads to high cytosolic glucose concentration in renal endothelial cells and pericytes with consequent biochemical dysfunction: activation of protein kinase C_β, hexosamine, and polyol pathways; metabolic pseudohypoxia; mitochondrial dysfunction and oxidative stress; and accumulation of advanced glycation end products (AGEs) (6). The link of high cytosolic glucose concentration to metabolic dysfunction was demonstrated by overexpression of the GLUT1 glucose transporter in renal mesangial cells that thereby acquired the characteristics of the diabetic phenotype, including increased extracellular matrix protein synthesis and activation of the polyol pathway (7). Supporting studies of mesangial and endothelial cells in hyperglycemic culture have exemplified the key features of biochemical dysfunction in hyperglycemia: the accumulation of triosephosphates (8), increased de novo synthesis of diacylglycerol and activation of protein kinase C_β (9), oxidative stress linked to mitochondrial dysfunction [sustained by high glycerophosphate shuttle activity (10)], concomitant activation of the hexosamine pathway, and the accumulation of methylglyoxal with increased formation of AGEs (11). Increased concentrations of triosephosphate glycolytic intermediates, glyceraldehyde-3-phosphate (GA3P), and dihydroxyacetonephosphate (DHAP) is the trigger for these processes (8,10). A pharmacological strategy that countered triosephosphate accumulation in hyperglycemia would suppress multiple pathogenic pathways and prevent the development of diabetic nephropathy. Activation of the reductive pentosephosphate pathway (PPP) by high-dose thiamine therapy may achieve this by increasing transketolase (TK) activity and stimulating the conversion of GA3P and fructose-6-phosphate (F6P) to ribose-5-phosphate (R5P) (Fig. 1). Supporting evidence for this intervention comes from studies showing the normalization of triosephosphates by activation of the reductive PPP in human erythrocytes in hyperglycemic culture by high-dose thiamine (12) and the correction of delayed replication, activation of protein kinase C (PKC), increased hexosamine and AGE concentrations, and oxidative stress in capillary and aortal endothelial cells in hyperglycemic culture by high-dose thiamine and S-benzoylthiamine monophosphate (benfotiamine) (13,14). We investigated the effect of high-dose thiamine and benfotiamine therapy on the development of incipient nephropathy in the streptozotocin (STZ)-induced diabetic rat model of diabetes with moderate insulin therapy. The minimum daily allow-

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Received for publication 21 March 2003 and accepted in revised form 8 May 2003.

AGE, advanced glycation end product; CEL, N_ε-(1-carboxyethyl)lysine; CML, N_ε-carboxymethyl-lysine; DHAP, dihydroxyacetonephosphate; F6P, fructose-6-phosphate; FL, fructosyl-lysine; GA3P, glyceraldehyde-3-phosphate; GFR, glomerular filtration rate; GSH, reduced glutathione; LC-MS/MS, liquid chromatography–triple quadruple mass spectrometry; MG-H1, methylglyoxal-derived hydroimidazolone N_δ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; MRM, multiple reaction monitoring; PKC, protein kinase C; PPP, pentosephosphate pathway; R5P, ribose-5-phosphate; RAGE, AGE receptor; RBC, red blood cell; STZ, streptozotocin; TK, transketolase; TMP, thiamine monophosphate; TPP, thiamine pyrophosphate.

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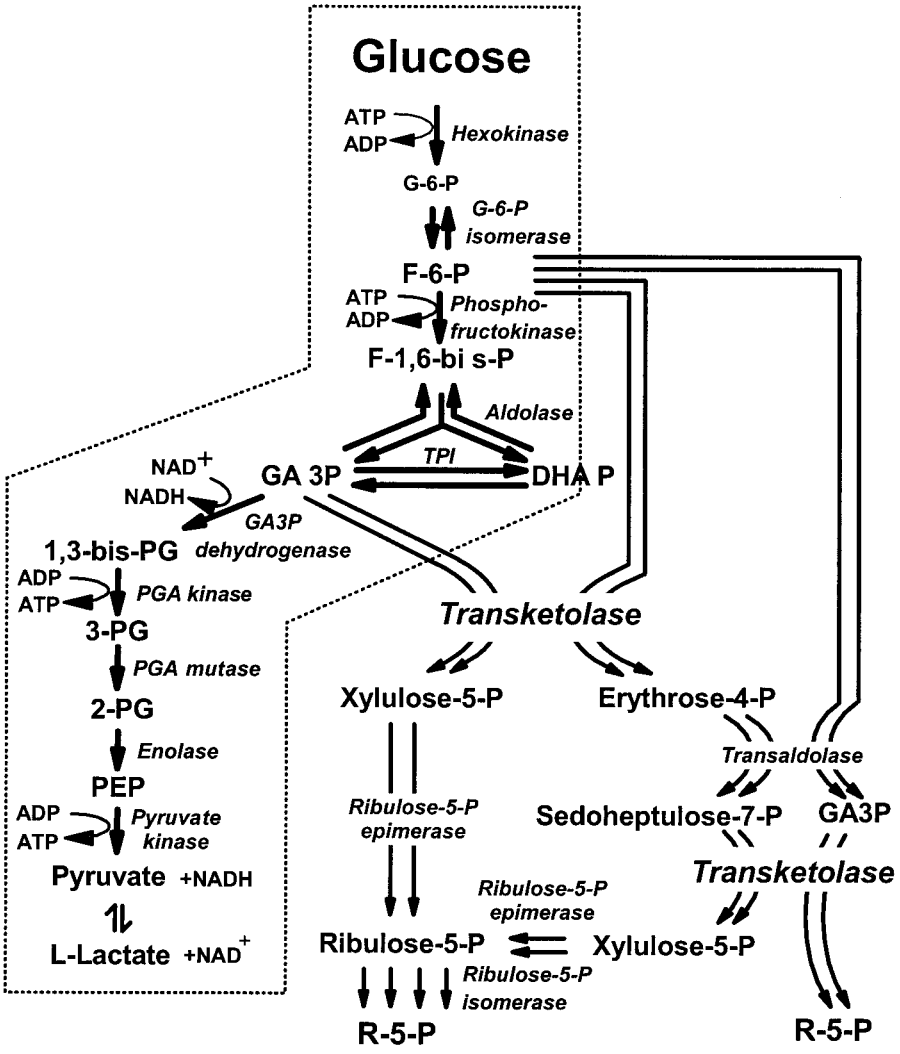

FIG. 1. Shunting of glycolytic intermediates from the Embden-Meyerhof pathway (dotted enclosure) to the reductive PPP in anaerobic glycolysis.

ance of thiamine for rats was 4 mg thiamine per kg diet (15). The concentration of thiamine in rat food in this study was 25.7 mg/kg, and hence normal control rats were not thiamine restricted. The conventional indicator of thiamine sufficiency is the "thiamine effect," the increase of TK activity with a saturating amount of exogenous TPP in ex vivo assay. When this increase is ≥15% of TK activity in the presence of saturating TPP, there is thiamine deficiency (16). In this study, incipient nephropathy developed over a 24-week period in the STZ diabetic rats, as judged by hyperfiltration and microalbuminuria, and both high-dose thiamine and benfotiamine therapy prevented it.

RESEARCH DESIGN AND METHODS

STZ diabetic rats. Male Sprague-Dawley rats, 250 g, were purchased from Charles River U.K. (Ramsgate, Kent, U.K.). They were kept two per cage at 21°C, 50–80% humidity, with daily 14-h light cycle, and had free access to food and water. Diabetes was induced by intravenous injection with 55 mg/kg STZ. Body weight and moderate hyperglycemia were stabilized by subcutaneous injection of 2 units Ultralente insulin every 2 days. Thiamine and benfotiamine were given orally, mixed with the food, at high doses (7 and 70 mg/kg daily) over 24 weeks to STZ diabetic and normal control rats. At 6-week intervals, venous blood samples (200 μl) were taken with heparin anticoagulant and 24-h urine samples were collected. Plasma glucose concentration was determined by glucose oxidase method, glycated hemoglobin HbA_1 by boronate affinity chromatography, and urinary and plasma creatinine by colorimetric assay (diagnostic kits 510, 442, and 555; Sigma). Glomerular filtration rate (GFR) was deduced as (urinary creatinine/plasma creatinine) × urine volume. The development of nephropathy was judged by the measurement of albuminuria and proteinuria. Urinary albumin was determined by SDS-PAGE (8% gels) of urine by calibrated densitometry of the albumin (66.5 kDa) band after Coomassie blue staining. The concentration of protein in the urine was determined by the Bradford method. Renal glomeruli were isolated by sieving of renal cortex tissue through 80- to 200-μm sieves and washed with 0.85% saline at 4°C (17). Where required, glomeruli were homogenized in 10 mmol/l sodium phosphate buffer, pH 7.4 and 4°C, and membranes sedimented by centrifugation (20,000*g*, 30 min, 4°C). All procedures were approved by the U.K. Home Office for work under the Animals (Scientific Procedures) Act 1986; project license 80/1481.

Metabolite and enzyme activity analysis. Thiamine and thiamine monophosphate (TMP) were determined in food, plasma, and urine by reversed-phase high-performance liquid chromatography with fluorimetric detection (18). TK activity was determined in glomerular extract and hemolysate by the method of Chamberlain et al. (19). For Western blotting, glomerular protein (150 μg/well) was separated by SDS-PAGE (10% gels), transferred to nitrocellulose and blocked with 5% milk protein, and blotted with rabbit anti-rat TK IgG (supplied by Prof. F. Paoletti) and anti-rabbit IgG-peroxidase conjugate, and blots were developed with enhanced chemiluminescence (AP Biotech, Amersham, U.K.).

GA3P and R5P were quantified in neutralized perchloric acid extracts of red blood cells (RBCs) by triple quadruple mass spectrometric detection (LC-MS/MS) using a Waters 2690 Separation module with a Micromass Quattro Ultima mass spectrometer (Waters-Micromass, Manchester, U.K.) (20). The column was Hypercarb (2.1 × 50 mm), the mobile phase was 12 mmol/l ammonium acetate, pH 4.8, and the flow rate was 0.2 ml/min. Analytes were detected by negative ion electrospray–mass spectrometric multiple reaction monitoring (MRM). The temperatures of the ion source and desolvation gas were 120°C and 350°C, respectively. The retention times, MRM transitions, collision energy, and fragment loss were GA3P 6.5 min, 168.7 >

151.4 Da, 8 eV, H_2O; R5P 9.0 min, 228.8 > 97.1 Da, 14 eV, ribose; and internal standard 2-deoxyglucose-6-phosphate 17.7 min, 243.2 > 97.1 Da, 16 eV, 2-deoxyglucose. The assay was calibrated with 50–500 pmol analytes and 250 pmol internal standard.

Glomerular PKC activity in situ was assayed with an epidermal growth factor receptor peptide fragment as substrate (21), VRKRTLRRL (Bachem, St Helens, U.K.). PKC activity was also assayed in membrane and particulate fractions with exogenous diacylglycerol (DAG; 1,2-dioleoyl-sn-glycerol) (22). The concentrations of glyoxal, methylglyoxal, 3-deoxyglucosone, and protein thiols in blood plasma were assayed as described (23,24).

Protein biomarker determination by LC-MS/MS. Fructosyl-lysine (FL) and AGEs were determined in enzymatic hydrolysates of glomerular protein by LC-MS/MS, calibrated by stable isotope substituted standards referenced to authentic analyte calibration curves. FL and AGEs—methylglyoxal-derived hydroimidazolone N_δ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), N_ε-(1-carboxyethyl)lysine (CEL), and N_ε-carboxymethyl-lysine (CML)—were determined. [*Guanidino*-$^{15}N_2$]-MG-H1 was prepared from [*guanidino*-$^{15}N_2$]-L-arginine after conversion to the N_α-t-BOC-derivative (25). [$^{13}C_6$]CEL and [$^{13}C_6$]CML were prepared from [$^{13}C_6$]L-lysine; [$^2H_4$]fructosyl-lysine was prepared from [4,4,5,5-$^2H_4$]L-lysine after conversion to the N_α-formyl derivative (26). Synthetic methods for the preparation, purification, and characterization of these AGE calibration standards were as described for their non–isotopically substituted analogs (27). Stable isotope-substituted standard amino acids were purchased from Cambridge Isotope Laboratories (Andover, MA). Glomerular protein (100 μg) was hydrolyzed exhaustively by consecutive incubation with pepsin, pronase E, and finally aminopeptidase and prolidase, as described (27). All steps at pH 7.4 were performed under nitrogen to prevent oxidation during the hydrolysis. Two 5-μm Hypercarb columns (Thermo Hypersil, Runcorn, U.K.) in series were used: 2.1 × 50 mm (column 1) and 2.1 × 250 mm (column 2). The mobile phase was 26 mmol/l ammonium formate, pH 3.8, with a two-step gradient of acetonitrile (17–25 min, 0–31% acetonitrile; 25–30 min, 31% acetonitrile; 30–35 min, 31–50% acetonitrile). The flow rate was 0.2 ml/min. The flow was diverted from column 2 at 22 min to facilitate elution of hydrophobic biomarkers. Flow from the column in the interval 4–35 min was directed to the MS/MS detector. Glycation markers were detected by electrospray positive ionization-mass spectrometric MRM. The ionization source temperature was 120°C and the desolvation gas temperature 350°C. The cone gas and desolvation gas flow rates were 150 and 550 l/h, respectively. The capillary voltage was 3.55 kV and the cone voltage 80 V. Argon gas (2.7×10^{-3} mbar) was in the collision cell and programmed molecular ion, fragment ion, and collision energies optimized to ±0.1 Da and ±1 eV for MRM detection. The amounts of internal standard used were 10 nmol for amino acids, 250 pmol for FL, and 10–50 pmol for glycation, oxidation, and nitrosation biomarkers. Limits of detection were 0.2–1 pmol, interbatch coefficients of variation 2–16% (n = 6), and analyte recoveries in the enzymatic hydrolysis >90% (except 71% for FL), depending on the analyte. The retention times, MRM transitions (molecular ion > fragment ion masses), collision energy, and fragment losses for analytes and calibration standards were lysine: 5.0 min, 147.1 > 84.3 Da, 15 eV, H_2CO_2 + NH_3, [$^{13}C_6$]lysine; CML: 6.7 min, 204.9 > 130.1 Da, 12 eV, $NH_2CH_2CO_2H$, [$^{13}C_6$]CML; CEL: 7.0 min, 219.2 > 130.1 Da, 13 eV, $NH_2CH(CH_3)CO_2H$, [$^{13}C_6$]CEL; FL: 7.0 min, 291.0 > 84.3 Da, 31 eV, H_2CO_2 + fructosylamine, [$^2H_4$]FL; arginine: 10.9 min, 175.2 > 70.3 Da, H_2CO_2 + $NH_2C(=NH)NH_2$, 15 eV, [$^{15}N_2$]arginine; and MG-H1: 23.6 and 24.0 min (two epimers), 229.2 > 114.3 Da, 14 eV, $NH_2CH(CO_2H)CH_2CH=CH_2$, [$^{15}N_2$]MG-H1.

Statistics. Data are means ± SE (n = 5–9). Significance of differences between mean and medians was assessed by Student's t test and the Mann-Whitney U test, as appropriate.

RESULTS

Thiamine and benfotiamine dosing of streptozotocin-induced diabetic rats with maintenance insulin therapy. Animal study groups in this investigation were as follows: normal controls (C), normal controls with high-dose (70 mg/kg per day) thiamine (CT70) and benfotiamine (CB70) therapy, diabetic controls (DC), and diabetic animals given high-dose thiamine (DT7 and DT70) and benfotiamine (DB7 and DB70) therapy (7 and 70 mg/kg per day). The mean food consumption of the rats is shown in Table 1. Hence, at baseline, dietary thiamine exceeded the recommended daily allowance by ~6-fold (C), 9-fold (D), 20-fold (DT7), and 140-fold (CT70 and DT70) and in thiamine equivalents, 20-fold (DB7) and 100-fold (CB70 and DB70). Mean body weights in the control groups increased from 268 and 235 g at baseline to 712 and 607 g after 24 weeks in thiamine and benfotiamine dosing studies, respectively; this was not changed significantly by thiamine and benfotiamine therapy. Mean body weights in the diabetic controls increased from 268 and 243 g at baseline to 351 and 318 g in thiamine and benfotiamine dosing studies, respectively; similar increases in mean body weight were found for diabetic rats with high-dose thiamine and benfotiamine therapy (Table 1). The diabetic rats had the characteristics of the diabetic state in both studies: increased plasma glucose concentration and increased glycated hemoglobin HbA_1. At 24 weeks, in the thiamine study, glycemic indicator values were as follows: plasma glucose concentration, diabetic 25.1 ± 4.3 versus control 6.0 ± 1.2 mmol/l ($P < 0.001$); and HbA_1, diabetic 17.7 ± 1.6% versus control 9.0 ± 0.8% ($P < 0.001$). At 24 weeks in the benfotiamine study, glycemic indicator values were as follows: blood glucose concentration, diabetic 31.8 ± 3.2 versus control 5.2 ± 1.4 mmol/l ($P < 0.001$); and HbA_1, diabetic 19.7 ± 2.0% versus control 8.7 ± 0.7% ($P < 0.001$). These increased levels in the diabetic rats were not changed by high-dose thiamine and benfotiamine therapy except for small decreases in HbA_1 of CT70 at 18 weeks, CB70 at 12 weeks, and CB70 and DB70 at 24 weeks with respect to normal and diabetic controls, respectively (Fig. 2).

TABLE 1
Body weight and food consumption of STZ diabetic and control rats with high-dose thiamine and benfotiamine therapy

Study group	Body weight at baseline (g)	Body weight at 24 weeks (g)	Mean study food consumption (g/day)
Thiamine dosing			
C	278 ± 13	712 ± 93*	36
CT70	265 ± 5	689 ± 32*	32
DC	268 ± 12	351 ± 41†‡	47§
CT7	266 ± 15	317 ± 30†‡	32‖
CT70	259 ± 15	398 ± 102†‡	36‖
Benfotiamine dosing			
C	235 ± 9	607 ± 32*	30
CB70	240 ± 11	675 ± 66*	30
DC	243 ± 15	318 ± 49†‡	54§
DB7	241 ± 8	380 ± 51†‡	62§¶
DB70	251 ± 8	334 ± 56†‡	46§#

*$P < 0.001$, †$P < 0.01$ vs. baseline control; ‡$P < 0.001$ vs. 24-week normal control; §$P < 0.001$ vs. normal control; ‖$P < 0.001$, ¶$P < 0.01$, #$P < 0.05$ vs. diabetic control.

Prevention of microalbuminuria and proteinuria by high-dose thiamine and benfotiamine. STZ diabetic rats on insulin maintenance therapy develop microalbuminuria over 24 weeks (28). Microalbuminuria was evident in the diabetic rats from 6 to 24 weeks, increasing from normoalbuminuria (2.0–2.2 mg albumin/24 h) to 12−17 mg/24 h at 6 weeks with a further progressive increase to 19–33 mg/24 h at 24 weeks ($P < 0.01$). Thiamine and benfotiamine therapy inhibited the development of microalbuminuria by 70–80% with no dose-response relationship evident ($P < 0.01$) except for benfotiamine at 6 weeks (Fig. 3*A* and *B*). Proteinuria showed similar changes, albeit with excreted amounts of protein, approx-

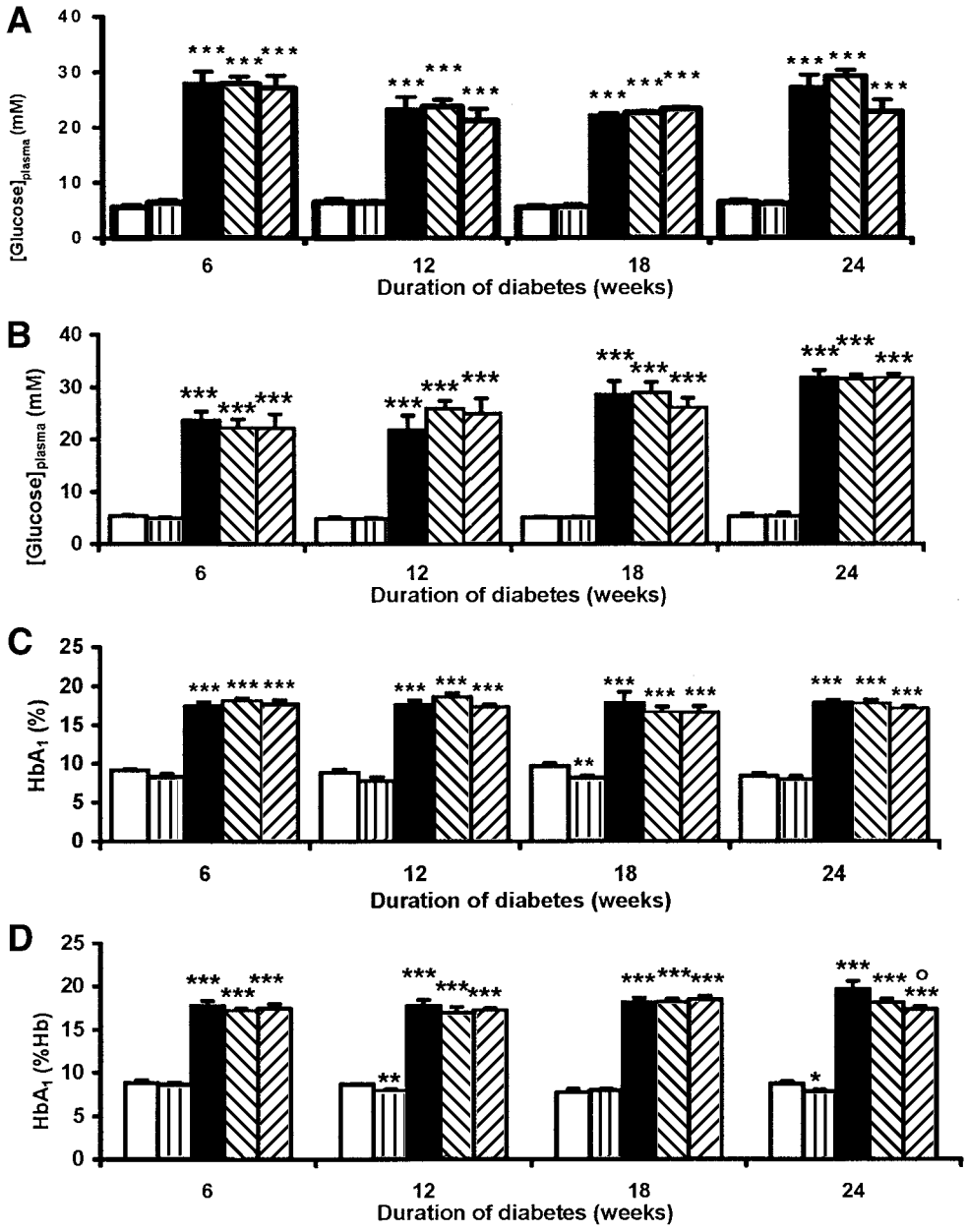

FIG. 2. Effect of high-dose thiamine and benfotiamine therapy on glycemic control in STZ diabetic rats and controls. Blood glucose concentration *A* and *B*: Glycated hemoglobin HbA_1 in thiamine and benfotiamine studies, respectively. *C* and *D*: Glycated hemoglobin HbA_1 in thiamine and benfotiamine studies, respectively. □, controls; ▥, control + 70 mg/kg thiamine or benfotiamine; ■, diabetic; ▧, diabetic + 7 mg/kg thiamine or benfotiamine; ▨, diabetic + 70 mg/kg thiamine or benfotiamine. Data are means ± SE (n = 5–9). *, o, and x denote significance with respect to control, diabetic control, and diabetic + 7 mg/kg thiamine derivative; one, two, and three symbols denote $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

imately fivefold higher than for intact albumin, except that a dose-response was generally evident (except at week 18) for thiamine therapy but not for benfotiamine therapy (except at 6 weeks) (Fig. 3*C* and *D*). Overall, there was no marked difference in potency between thiamine and benfotiamine.

Hyperfiltration developed in the diabetic controls. The increase in GFR in STZ diabetic rats was not decreased by thiamine, but it was prevented by benfotiamine at 6–18 weeks. At 24 weeks of benfotiamine therapy, mild hyperfiltration had developed in the diabetic rats (Fig. 4). A decline in GFR was not observed, as is typical of this experimental model.

Thiamine status, transketolase activity and expression, and activation of the reductive PPP. We assessed the effect of high-dose thiamine and benfotiamine therapy on thiamine status of the study group rats by measuring the plasma concentrations of thiamine and TMP; thiamine pyrophosphate (TPP) was not detectable in blood plasma. We discovered the previously unrecognized characteristic of STZ diabetic rats, that they are thiamine deficient; the plasma thiamine concentration was decreased 69% in diabetic rats in the thiamine dosing study and 48% in the benfotiamine dosing study, with respect to normal controls. This was due to increased urinary excretion of thiamine. In the thiamine dosing study, urinary thiamine excretion was 0.55 μmol/24 h in controls and 2.28 μmol/24 h in diabetic rats ($P < 0.01$) and in the benfotiamine dosing study, 0.35 μmol/24 h in controls and 2.27 μmol/24 h in diabetic rats ($P < 0.01$). There was an associated eightfold increase in the renal clearance of thiamine in STZ diabetic rats, with respect to controls (thiamine study, 0.51 vs. 4.03 ml/min, $P < 0.01$; and benfotiamine study, 0.38 vs. 3.01 ml/min, $P < 0.01$). This was prevented by 70 mg/kg thiamine and 7 mg/kg benfotiamine but not by 7 mg/kg thiamine. High-dose thiamine and benfotiamine therapy gave a dose-dependent increase in the plasma concentration of thiamine: 7 mg/kg thiamine normalized and 70

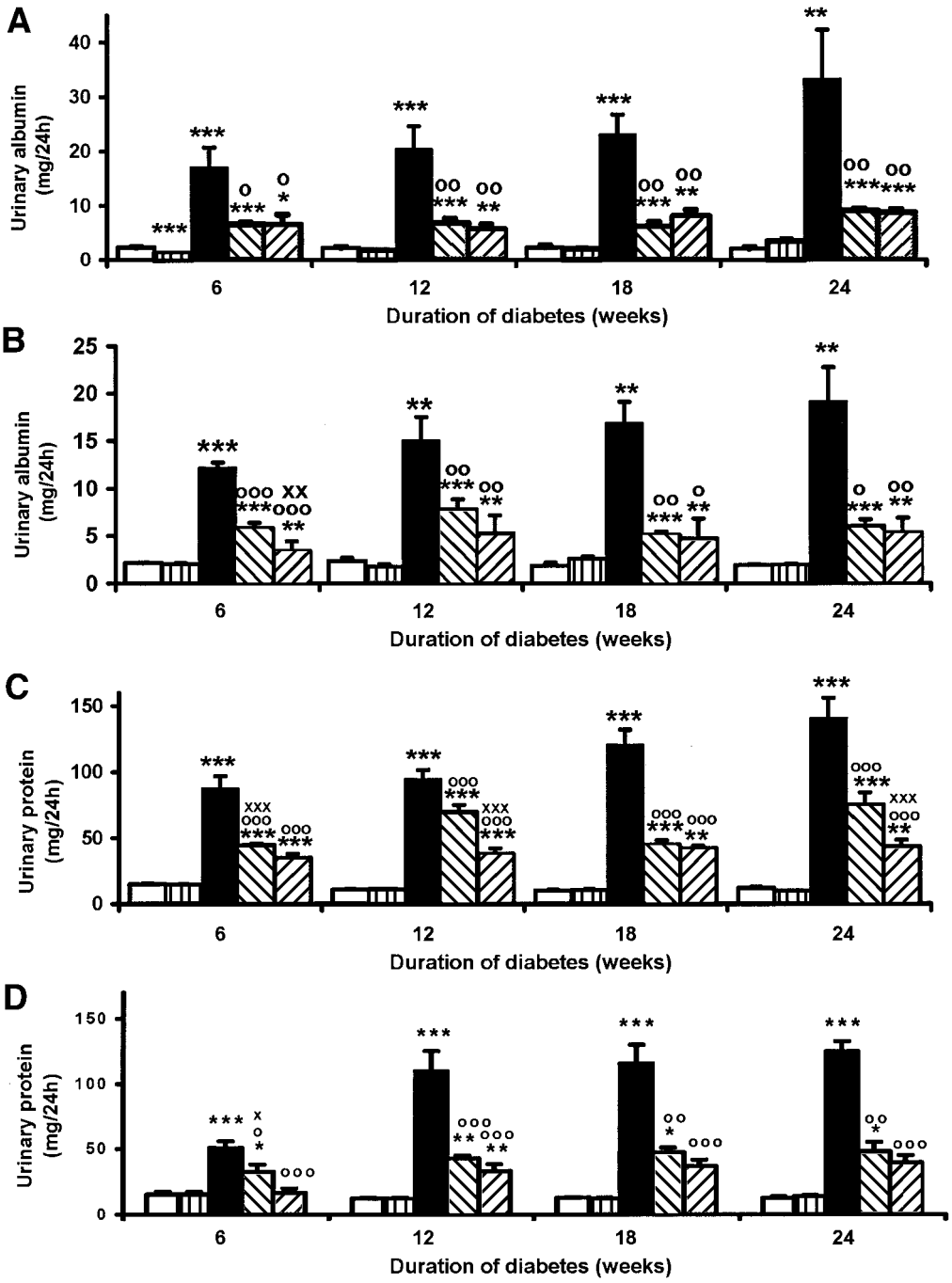

FIG. 3. Effect of high-dose thiamine and benfotiamine therapy on renal function in STZ diabetic rats and controls. Urinary albumin (*A* and *B*) and urinary proteins (*C* and *D*) in thiamine and benfotiamine studies, respectively. □, controls; ▥, control + 70 mg/kg thiamine or benfotiamine; ■, diabetic; ▧, diabetic + 7 mg/kg thiamine or benfotiamine; ▨, diabetic + 70 mg/kg thiamine or benfotiamine. Data are means ± SE (*n* = 5–9).

mg/kg thiamine supranormalized plasma thiamine levels in diabetic rats (Fig. 5*A*); and both 7 and 70 mg/kg benfotiamine supranormalized plasma thiamine levels (Fig. 5*B*). Benfotiamine did not increase the plasma concentration of TMP significantly in control or STZ diabetic rats.

We determined the effect of increased availability of thiamine on glomerular TK activity. Diabetic rats had decreased TK activity (thiamine study −29%, $P < 0.05$; benfotiamine study −24%, $P < 0.001$), which was normalized by thiamine and benfotiamine therapy (Fig. 5*C* and *D*). There was also a significant but small "thiamine effect" on glomerular TK activity in diabetic rats only: thiamine study 7.4 ± 3.3% and benfotiamine study 1.9 ± 1.0% ($P < 0.05$). Decrease in TPP cofactor availability, however, as assessed by the "thiamine effect," did not account for most of the decreased glomerular TK activity in diabetic rats and reversal of this by high-dose thiamine and benfotiamine therapy. Rather, TK expression was decreased in diabetic rats and supranormalized in thiamine- and benfotiamine-treated control and diabetic rats (Fig. 5*E–G*).

Decreased TK activity was associated with a decreased R5P/GA3P concentration ratio that was increased significantly in both control and diabetic rats by thiamine therapy. The R5P/GA3P concentration ratio (mean ± SE) in RBCs was C 0.91 ± 0.19, CT70 3.05 ± 0.78 ($P < 0.05$ vs. normal control), DC 0.51 ± 0.15, DT7 2.12 ± 0.55 ($P < 0.05$), and DT70 1.71 ± 0.41 ($P < 0.05$ vs. diabetic control).

Prevention of diabetes-associated biochemical dysfunction by high-dose thiamine and benfotiamine therapy. We next sought to assess if activation of the reductive

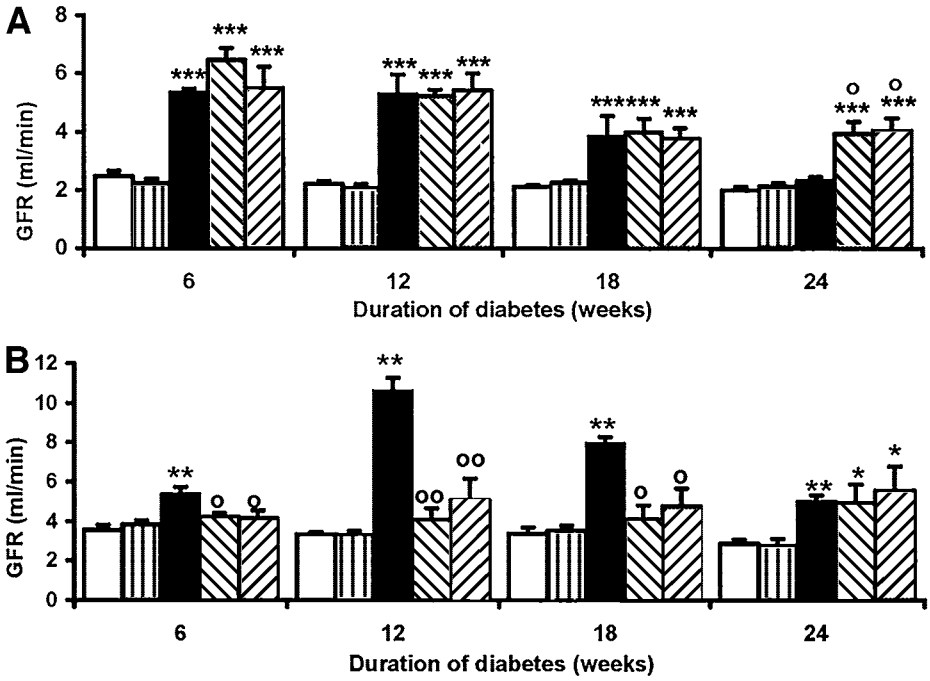

FIG. 4. Effect of high-dose thiamine and benfotiamine therapy on GFR in STZ diabetic rats and controls. *A* and *B*: GFR in thiamine and benfotiamine studies, respectively. □, controls; ▥, control + 70 mg/kg thiamine or benfotiamine; ■, diabetic; ▧, diabetic + 7 mg/kg thiamine or benfotiamine; ▨, diabetic + 70 mg/kg thiamine or benfotiamine. Data are means ± SE (n = 5–9).

PPP by thiamine and benfotiamine had countered biochemical dysfunction associated with the development of diabetic nephropathy—activation of PKC, dicarbonyl stress, glycation, and oxidative stress. Using an assay of in situ PKC activity, we found increased glomerular PKC activity in STZ diabetic rats, with respect to controls (thiamine dosing study 38 ± 3% and benfotiamine dosing study 54 ± 6%). This was reversed in a dose-dependent manner by thiamine and benfotiamine therapy (Fig. 6*A* and *B*). Specific PKC activities in cytosolic and membrane fractions of glomeruli stimulated with exogenous DAG were also determined. Cytosolic and membrane fraction PKC activities were increased by approximately onefold in diabetic controls, and these increases were reversed by 55 and 66% with 7 and 70 mg/kg thiamine (Fig. 6*C* and *E*). Similar effects were found for benfotiamine therapy (Fig. 6*D* and *F*). Neither high-dose thiamine nor benfotiamine completely reversed diabetes-induced increases in glomerular PKC activity.

Dicarbonyl compounds such as methylglyoxal, glyoxal, and 3-deoxyglucosone are implicated in carbonyl stress and in increased formation of AGEs in diabetes and are linked to the development of diabetic nephropathy (29–31). Methylglyoxal concentration is increased by the degradation of abnormally high concentrations of GA3P and DHAP in cells suffering cytosolic hyperglycemia (32). High-dose thiamine therapy was therefore expected to decrease the plasma concentration of methylglyoxal, and this was indeed found. Glyoxal and 3-deoxyglucosone were also increased in the plasma of STZ diabetic rats, and surprisingly, the increased plasma concentrations of these glycating agents were also decreased by thiamine (Fig. 7*A*).

We determined markers of glycation (FL and AGEs in glomerular protein) and oxidative stress (plasma protein thiols) in STZ diabetic rats with high-dose thiamine and benfotiamine therapy. The methylglyoxal-derived AGEs MG-H1 and CEL were increased approximately twofold in glomerular protein of diabetic rats and normalized in a dose-dependent manner by thiamine and benfotiamine (Fig. 7*B–E*). There were smaller increases in CML in diabetic controls of borderline significance (thiamine study 0.50 ± 0.19 vs. 0.27 ± 0.11 mmol/mol lysine and benfotiamine study 0.42 ± 0.17 vs. 0.26 ± 0.12 mmol/mol lysine; $P < 0.05$). These increases were partially reversed by thiamine and benfotiamine therapy. Glomerular protein FL was increased in diabetic controls but was not decreased by thiamine and benfotiamine therapy. The concentration of plasma thiols was measured as a marker of oxidative stress. Plasma protein thiols were decreased in STZ diabetic rats by 30% of control levels in both thiamine and benfotiamine dosing studies. Benfotiamine therapy at 50 mg/kg reversed this decrease significantly; thiamine (7 and 70 mg/kg) and benfotiamine at 7 mg/kg did not (Fig. 7*F* and *G*).

DISCUSSION

Investigating the effect of high-dose thiamine and benfotiamine therapy on STZ diabetic rats to prevent the development of nephropathy, we found that STZ diabetic rats had abnormally low plasma thiamine concentration and glomerular TK activity, although they were not thiamine deficient by the "thiamine effect" criterion (16). Plasma thiamine was decreased in diabetic rats by increased renal clearance. This was associated with increased urinary excretion of thiamine. Excessive diuresis of the diabetic rats (increased 14-fold) and decreased thiamine reabsorption in renal tubules are probable causes. Thiamine in the glomerular filtrate is reabsorbed in the proximal tubules by a thiamine/H^+ antiport activity (33). Decreased thiamine reabsorption may be due to structural damage in the proximal tubular cells or metabolic derangements (glucosuria, intracellular acidosis, or other effects).

TK expression is decreased by thiamine deficiency (34), and this was found for the diabetic control rats in this

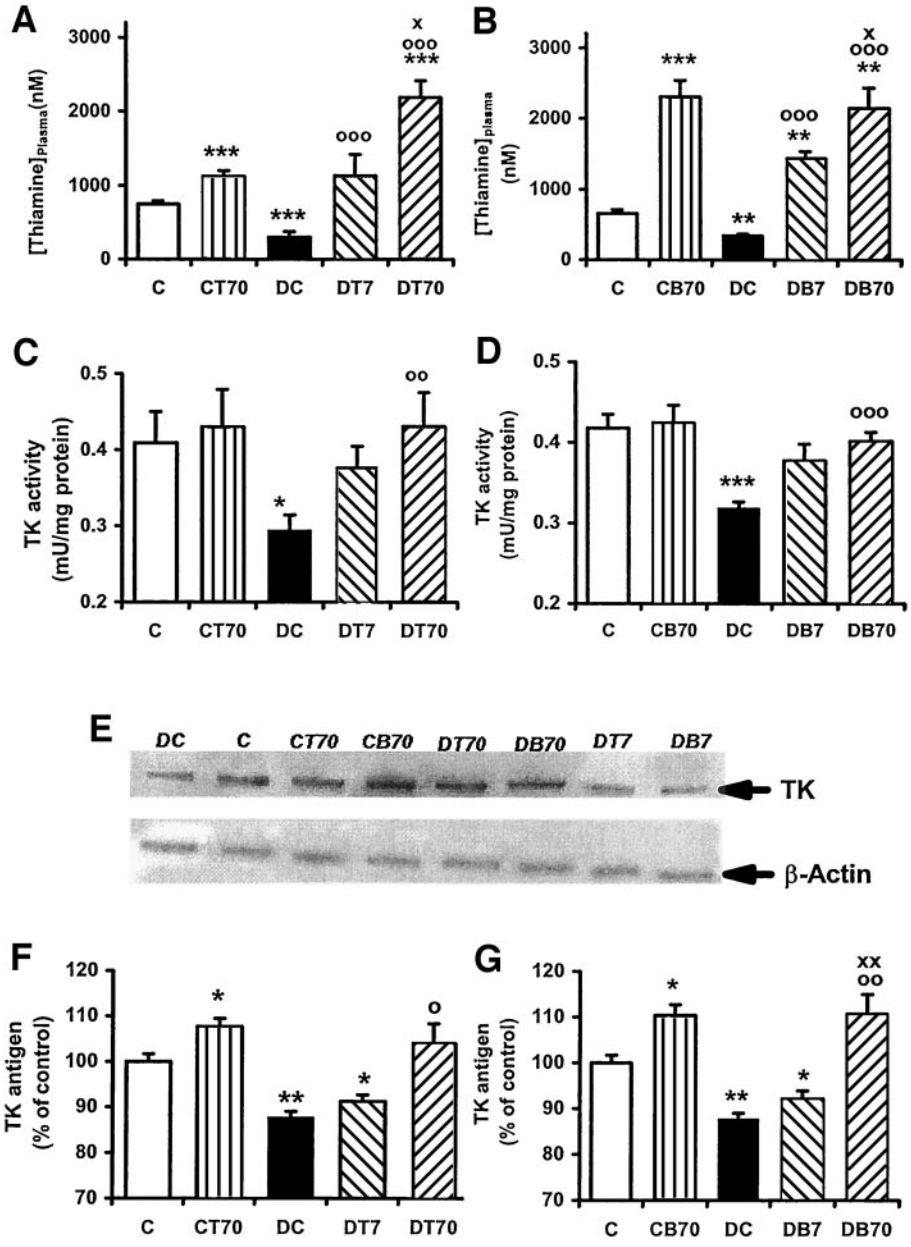

FIG. 5. Effect of high-dose thiamine and benfotiamine therapy on thiamine status and TK expression in STZ diabetic rats and controls. Plasma thiamine concentration (*A* and *B*), TK activity (*C* and *D*), and TK expression (*F* and *G*) in high-dose thiamine and benfotiamine studies, respectively. *E*: Western blot and β-actin control. □, control; ▥, control + 70 mg/kg thiamine or benfotiamine; ■, diabetic; ▧, diabetic + 7 mg/kg thiamine or benfotiamine; ▨, diabetic + 70 mg/kg thamine or benfotiamine. Data are means ± SE (n = 5–9).

study. This contributed to the decrease in glomerular TK activity in diabetic rats. High-dose benfotiamine therapy increased plasma thiamine levels in both control and diabetic rats. It prompted higher increases in plasma thiamine concentrations of normal control and diabetic rats with the 7-mg/kg dose than thiamine. Both high-dose thiamine and benfotiamine normalized glomerular TK activity and reversed the decreased expression of TK in diabetic rats. Recently, high-dose benfotiamine therapy (80 mg/kg daily) was found to also increase the activity of TK in the retina of Wistar diabetic rats, but no decrease in TK was found in diabetic controls (35). Clinical diabetes is also associated with a mild thiamine deficiency. Two studies found 18 and 76%, respectively, of diabetic subjects studied to have plasma thiamine concentrations lower than the normal range minimum (36,37). In the latter study, with moderate glycated hemoglobin (9% Hb), only 21% of diabetic patients had RBC TK activity higher than the normal range minimum, and the mean thiamine effect was 17%, indicating borderline thiamine deficiency (37). Our study suggests that such thiamine deficiency may exacerbate the risk of developing nephropathy.

In diabetic rats, high-dose thiamine and benfotiamine therapy prevented the development of microalbuminuria and proteinuria. There was no clear evidence of a dose-response relationship except for proteinuria for high-dose thiamine therapy. High-dose thiamine and benfotiamine therefore prevented incipient nephropathy. Whether they also prevent the progression from incipient to overt nephropathy remains to be investigated.

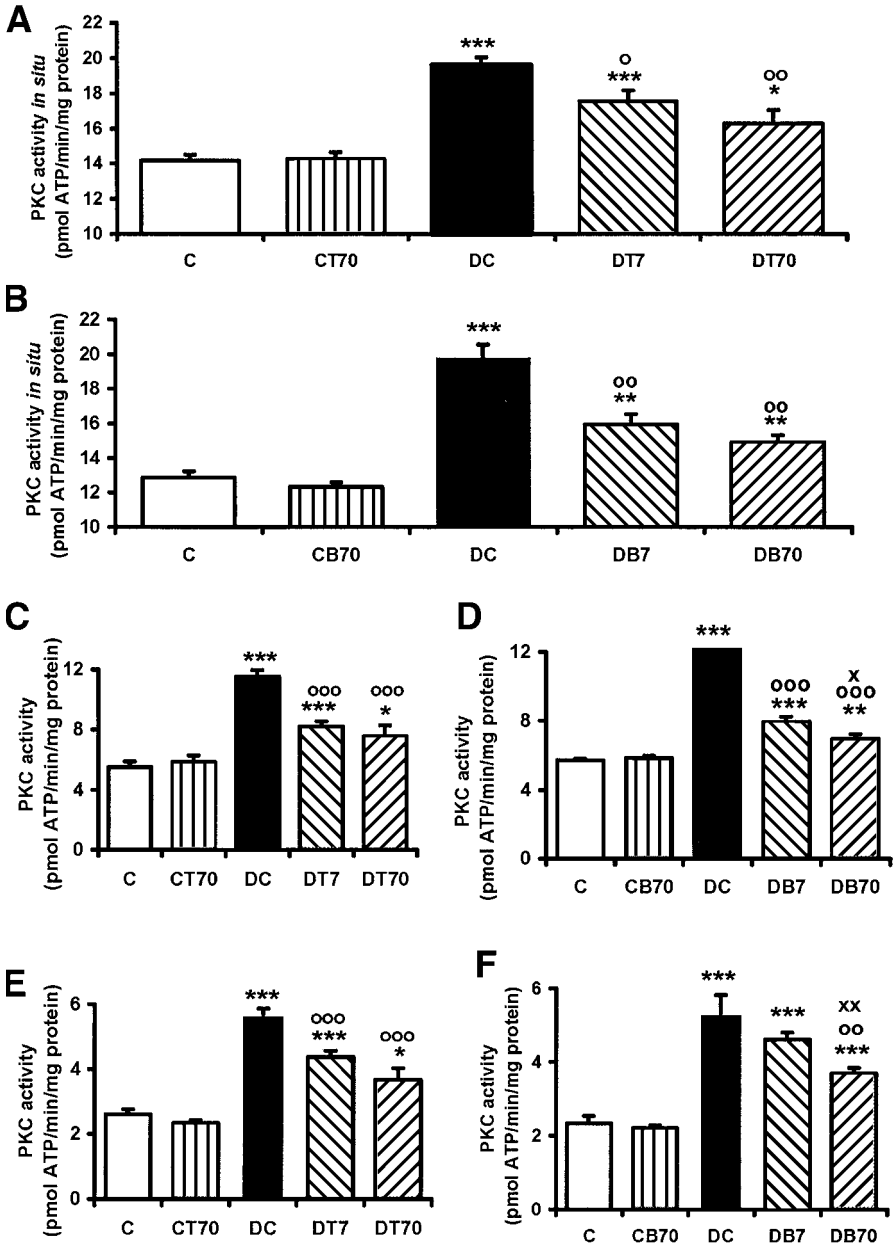

FIG. 6. Effect of high-dose thiamine and benfotiamine therapy on renal glomerular PKC activity in STZ diabetic rats and controls. PKC activity in situ (*A* and *B*), cytosolic-specific PKC activity (*C* and *D*), and membrane-specific PKC activity (*E* and *F*) in high-dose thiamine and benfotiamine studies, respectively. □, control; ▥, control + 70 mg/kg thiamine or benfotiamine; ■, diabetic; ▧, diabetic + 7 mg/kg thiamine or benfotiamine; ▨, diabetic + 70 mg/kg thiamine or benfotiamine. Data are means ± SE (n = 5–9).

Activation of PKC_β in renal glomeruli has been linked to development of diabetic nephropathy. In renal glomeruli, activation of PKC_β stimulated the expression of vascular endothelial growth factor, transforming growth factor-β, and extracellular matrix components such as type IV collagen (38). PKC activation in cytosolic hyperglycemia occurred via increased de novo synthesis of diacylglycerol from DHAP via glycerol-3-phosphate and stepwise acylation. We found increased glomerular PKC activity in situ and specific activities in cytosolic and membrane fractions of renal glomeruli of diabetic controls. Both high-dose thiamine and benfotiamine markedly suppressed PKC activation to a similar extent. These changes reflect increased expression of glomerular PKC in STZ-induced diabetes (38), and suppression of this by high-dose thiamine repletion is a likely contributory factor to the prevention of incipient nephropathy.

Protein glycation occurs via direct reaction of glucose with lysine and NH_2-terminal amino groups of proteins to form FL and other fructosamines that may degrade to form AGEs, and also by the reaction of α-oxoaldehydes with proteins to directly form lysine and arginine residue-derived AGEs (39). High-dose thiamine repletion prevented the diabetes-associated accumulation of methylglyoxal. It may do this by preventing triosephosphate accumulation that leads to increased formation of methylglyoxal. The accumulation of glyoxal and 3-deoxyglucosone was also decreased by high-dose thiamine repletion, however, probably by maintenance of high levels of NADPH and reduced glutathione (GSH) in tissues to sustain their rapid metabolism by NADPH-dependent 3-deoxyglucosone reductase and the GSH-dependent glyoxalase system, which also detoxifies methylglyoxal (40). We found that the AGEs MG-H1 and CEL were markedly increased, and CML increased moderately, in glomerular protein of STZ diabetic rats; high-dose thiamine and benfotiamine both suppressed AGE accumulation to a similar extent. MG-H1, and similar hydroimidazolones derived from 3-deoxygluocosone and

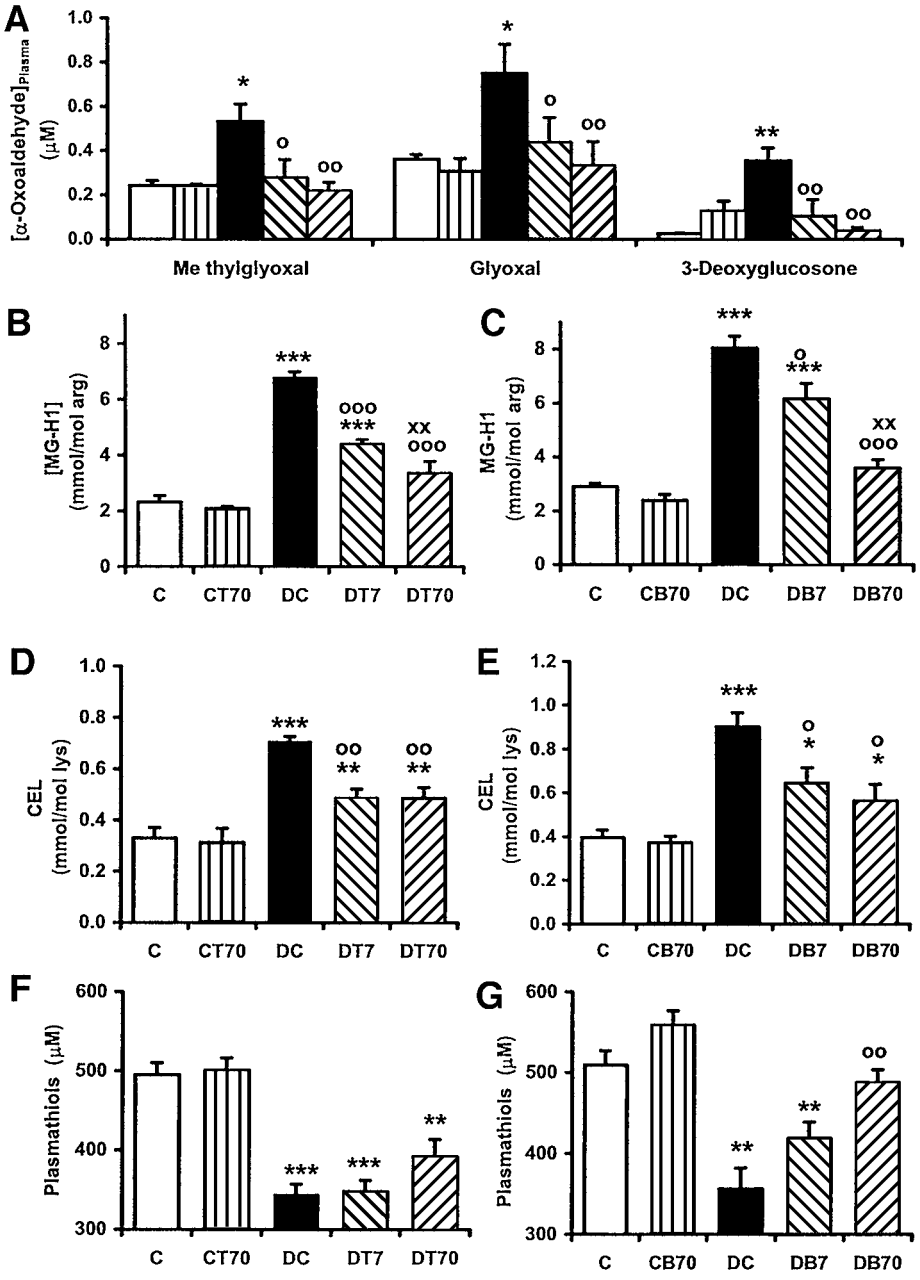

FIG. 7. Effect of high-dose thiamine and benfotiamine therapy on dicarbonyl stress, glomerular AGEs, and oxidative stress in STZ diabetic rats and controls. Dicarbonyl stress: Plasma α-oxoaldehydes in the high dose thiamine study (*A*); glomerular AGEs: MG-H1 (*B* and *C*) and CEL (*D* and *E*) in high-dose thiamine and benfotiamine studies, respectively. Oxidative stress plasma thiols (*F* and *G*) in high-dose thiamine and benfotiamine studies, respectively. □, control; ▥, control + 70 mg/kg thiamine or benfotiamine; ■, diabetic; ▧, diabetic + 7 mg/kg thiamine or benfotiamine; ▨, diabetic + 70 mg/kg thiamine or benfotiamine. Data are means ± SE (*n* = 5–9).

glyoxal, were major AGEs found in glomerular protein. All were increased in STZ diabetic rats, and the increases were reversed in a dose-dependent manner by thiamine and benfotiamine therapy (data not shown). CML and hydroimidazolones are thought to bind specifically to AGE receptors (RAGEs) of glomerular endothelial cells, pericytes, and podocytes (41). Activation of RAGE in renal glomeruli is implicated in growth factor expression, leading to mesangial expansion and glomerulosclerosis in incipient nephropathy. Suppression of AGE accumulation may contribute to the prevention of incipient nephropathy by high-dose thiamine repletion (42).

We examined plasma protein thiols as an indicator of oxidative stress. They were consistently decreased in the diabetic controls of both thiamine and benfotiamine studies, but only treatment with 70 mg/kg benfotiamine prevented this decrease. Because 7 mg/kg benfotiamine and both doses of thiamine prevented the development of proteinuria, high-dose thiamine repletion prevented incipient nephropathy in STZ diabetic rats without obligatory concomitant suppression of oxidative stress.

These observations suggest that high-dose thiamine repletion suppressed the development of incipient nephropathy in experimental diabetes in which effects on the PKC, glycation, and oxidative stress pathways were involved. We found similar effects on human mesangial cells in hyperglycemic culture in vitro (R.B.-J., N.K., N.A., S.B., P.J.T., unpublished data). The primary intervention was the prevention of thiamine deficiency and induction of TK expression with consequent activation of the reductive PPP shunt. It is remarkable that these effects were achieved by increasing the dietary availability of thiamine to diabetic rats by as little as 20 times the minimum daily allowance, although this was sufficient to prevent thiamine deficiency. Thiamine deficiency exacerbated the development of diabetic nephropathy. We therefore propose that

clinical diabetic subjects should avoid becoming thiamine deficient, even weakly so, and that high-dose thiamine repletion should be considered for therapy to prevent the development of clinical diabetic nephropathy.

ACKNOWLEDGMENTS

We thank the Juvenile Diabetes Research Fund and the Wellcome Trust (U.K.) for support for research. We also thank Professor F. Paoletti, Department of Experimental Pathology and Oncology, University of Florence, Italy, for the gift of polyclonal antibodies to rat transketolase.

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