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Research Article

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Impaired Nitric Oxide–dependent Cyclic Guanosine Monophosphate Generation in Glomeruli from Diabetic Rats

Evidence for Protein Kinase C–mediated Suppression of the Cholinergic Response

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Abstract

Nitric oxide (NO)-dependent cyclic guanosine monophosphate (cGMP) generation was examined in glomeruli isolated from 1–2-wk and 2-mo streptozotocin diabetic (D) and control (C) rats. After 1–2 wk of diabetes, ex vivo basal cGMP generation and cGMP responses to carbamylcholine (CCh) were significantly suppressed in glomeruli from D compared with those from C, whereas cGMP responses to the calcium ionophore A23187 and nitroprusside (NP) did not differ in glomeruli from D vs. those from C. After 2 mo, glomeruli from D did not respond to CCh, and responses to A23187 and NP were suppressed compared with those from C. Differences in basal, CCh, and A23187-responsive cGMP between D and C were abolished by the NO synthetase inhibitor N^G-monomethyl-L-arginine. Soluble glomerular guanylate cyclase prepared from either D or C responded indistinguishably to NP, suggesting a role for NO quenching in the suppression of cGMP in intact glomeruli from D. Compared with those from C, glomeruli isolated from D demonstrated increased generation of thromboxane A_2 (TXA_2) and activation of protein kinase C (PKC). Both the TXA_2/endoperoxide receptor antagonist Bay U3405 and inhibitors of PKC activity restored a cGMP response to CCh in glomeruli from D. Conversely, in glomeruli from C, the TXA_2/endoperoxide analogue U46619 activated PKC and suppressed the cGMP response to CCh. Both of those actions were blocked by inhibitors of PKC. The results indicate a progressive impairment of NO-dependent cGMP generation in glomeruli from D which may be mediated in part by TXA_2 and activation of PKC. This impairment may participate in glomerular injury in diabetes. (*J. Clin. Invest.* 1994. 93:311–320.) Key words: cyclic guanosine monophosphate•diabetic glomerulosclerosis•nitric oxide•protein kinase C•streptozotocin diabetes

Introduction

The endothelial derived relaxing factor nitric oxide (NO), whose cellular actions are expressed through activation of the heme-dependent cytosolic guanylate cyclase–cGMP system (1), has been shown to modulate glomerular capillary pressure (2, 3), mediate the renal vasodilatory response to arginine (4) and cholinergic agents (5), and antagonize the actions of vasoconstrictor hormones on glomerular mesangial cells (6). Chronic suppression of NO production in the rat results in sustained increases in systemic and intraglomerular capillary pressure, and is accompanied by the development of proteinuria and glomerular sclerotic injury (7). The impact of diabetes mellitus on NO production and on the expression of its cellular actions remains unclear. The role, if any, of altered NO production or cellular actions in the pathogenesis of the functional and structural changes in the glomerulus in diabetes is also uncertain. Some observations have implicated enhanced generation or actions of NO in the pathogenesis of glomerular hyperfiltration and vascular dysfunction in diabetes. In this regard, the ability of aminoguanidine to prevent the increase in vascular permeability to albumin seen in early diabetes in the rat was ascribed to suppression of NO-synthetase activity (8, 9). In a preliminary report, administration of the NO-synthetase inhibitor *N*-nitro-L-arginine methyl ester was found to suppress glomerular hyperfiltration in diabetic rats, and to eliminate differences in the autoregulation of renal blood flow between diabetic and control rats (10). By contrast, Kiff et al. (11), in studies with NAME found no evidence that vasodilation of the renal and mesenteric vascular beds of diabetic rats was linked to increased production or actions of NO, and concluded that NO production and/or cellular actions in these vascular beds were either unchanged or decreased in diabetic compared to control rats. In this regard, a blunted vasodilatory response to cholinergic agents has been repeatedly documented in the aorta of diabetic rats (5, 12–17). Moreover, the cGMP responses to both carbamylcholine (CCh)[1] and nitroprusside (NP) were recently reported to be reduced in glomeruli isolated from diabetic rats (18). In view of the effects of chronic suppression of NO production on glomerular function and structure in nondiabetic rats (7), impaired glomerular generation of NO, impaired expression of the action of NO to increase cGMP, or both might contribute to glomerular capillary hypertension in diabetes, and thereby to the pathogenesis of glomerular injury in this disorder (19).

In the present study, we examined cGMP generation in glomeruli isolated from the streptozotocin diabetic rat model in response to CCh and the calcium ionophore A23187. These agents increase cGMP by activation of the calcium-calmodulin–regulated, constitutive NO–synthetase system with consequent increased endogenous formation of NO and stimulation of NO-responsive cytosolic guanylate cyclase (20). The capacity of these two agents to stimulate cGMP formation was compared to that of NP which acts as an exogenous NO generating system, and atrial natiuretic peptide which increases cGMP by receptor-mediated, non–NO-dependent activation of membranous guanylate cyclase. The results indicate that in the glomerulus diabetes initially impairs basal and CCh-induced NO-

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1. *Abbreviations used in this paper:* ANP, atrial natriuretic protein; C and D, nondiabetic control and diabetic (rats); CCh, carbamylcholine; MARCKS, myrisoylated, alanine-rich C kinase substrate; NMA, N^G-monomethyl-L-argine; NP, nitroprusside; PKC, protein kinase C.

dependent cGMP generation and subsequently also suppresses the actions of exogenous NO to increase cGMP. Because we have previously shown that the protein kinase C (PKC) system is activated in glomeruli isolated from diabetic rats (21, 22), and because activation of PKC has been implicated in suppression of NO generation and/or cellular actions (23–26), the potential role of PKC in mediating the changes in NO-dependent increases in cGMP in glomeruli from diabetes was also examined.

Methods

Female Sprague Dawley rats (180–200 g, Zivic Miller Laboratories, Pittsburgh, PA) were age and weight matched and placed in one of six groups: group I, 1–2-wk diabetic; group II, 1–2-wk control; group III, 1–2-wk diabetic treated with insulin; group IV, 2-mo diabetic; group V, 2-mo control, and group VI, 2-mo diabetic treated with insulin. Diabetic rats received 60 mg/kg streptozotocin in sterile 0.010 M citric acid in saline, pH 4, by tail vein. Only streptozotocin-treated diabetic rats with blood glucose higher than 300 mg/dl glucose 3 d after injection of streptozotocin were included in the study. Groups IV and VI received a dose of insulin (10 U regular insulin/kg per d by osmotic mini-pump) which maintained glucose at near normal levels, as previously described (21). Blood glucose was determined from tail vein blood with a blood glucometer (Diascan-S; Home Diagnostics, Inc., Eatontown, NJ), 3 d after injection of streptozotocin, every third day for the first 1–2 wk, and then weekly thereafter.

Isolation and incubation of glomeruli. For each experimental condition shown in the figures, glomeruli were isolated from the kidneys of five rats from one study group, as previously described (21). Glomeruli were washed, suspended in KRB buffer (pH 7.4) containing 5 mM glucose (KRBG) and 2 mM 1-methyl-3-isobutylmethylxanthine. The phosphodiesterase inhibitor was routinely included to inhibit cGMP degradation, and thus to permit assessment of changes in cGMP synthesis. The suspension was then aliquoted (0.5 ml per well of a 24-well plate) and incubated at 37°C with shaking in a plexiglass chamber in an atmosphere of 95% O_2, 5% CO_2 under the conditions indicated in the text. At the end of the incubations, 0.5 ml of pH 6.0 Na acetate at 95°C was added to the glomeruli plus media, and the incubates were transferred to a grinding vessel. The well was washed twice with additional 0.5-ml aliquots of pH 6.0 Na acetate at 95°C, and the washes were added to the extract. The extracts were homogenized and centrifuged at 1,000 *g* for 15 min. The supernatant was used for assay of cGMP. The glomerular pellet was extracted in 1 N NaOH and used for determination of protein by the Bradford method (27). cGMP was assayed as previously reported (28) using a radioimmunoassay kit obtained from Dupont New England Nuclear, Boston, MA. SOD from human erythrocytes (2,000–4,000 U/mg protein) was employed in some incubations and was purchased from Sigma Chemical Co., St. Louis, MO.

In situ phosphorylation of the PKC-specific 80,000-M_r substrate. Glomeruli were isolated from the kidneys of five rats from each study group, washed, and resuspended in phosphate free minimum essential medium with 30 μCi/0.5 ml [^{32}P] orthophosphate. The glomerular suspension was aliquoted (0.5 ml per well of a 24-well plate) and incubated for 3 h at 37°C with shaking in a plexiglass chamber equilibrated with 95% O_2, 5% CO_2. Test agents were added at the concentrations and for the times indicated in the text. Endogenous phosphorylation was terminated by washing the glomeruli in KRBG containing 2 mM phosphate. The 80,000-M_r protein substrate of PKC was extracted in 2.5% perchloric acid plus 1 mM PMSF as previously described (29). After 20 min on ice the perchloric acid–soluble fraction was isolated by centrifugation at 13,500 *g* for 20 min. The supernatant was brought to 20% trichloroacetic acid, 10 μg of bovine serum albumin was added, and the 80,000 M_r protein was isolated by centrifugation for 20 min at 13,500 *g*. The precipitated proteins were solubilized in 3% SDS, 10 mM Tris, pH 7.8, 5% glycerol, 20% β-mercaptoethanol, 1 mM PMSF, and separated electrophoretically on 8% SDS-polyacrylamide gels by the method of Laemmli (30). In situ phosphorylation was quantitated by densitometry of the autoradiograms and the results normalized to the density of a 40,000-M_r protein whose density was not affected by any of the test agents employed in the present study.

Identification of the 80,000-M_r protein as belonging to the myristoylated, alanine-rich C kinase substrate (MARCKS) family of proteins was verified by extraction and immunoprecipitation with antibodies raised against the amino-terminal region of the MARCKS protein, as originally described by Lobaugh and Blackshear (31) and previously reported by our laboratory (29). In studies comparing in situ phosphorylation of the 80,000-M_r substrate in glomeruli isolated from diabetic compared to control rats, the specific activity (μCi/μmol) of [^{32}P]ATP in the two glomerular preparations was assessed after labeling with [$^{32}P_i$]. This was determined from cAMP-dependent protein kinase catalyzed ^{32}P incorporation into a standard quantity of histone, according to the procedures of Hawkins et al. (32).

Assay of guanylate cyclase activity. Guanylate cyclase activity in 100,000-*g* soluble fractions of glomerular homogenates was assayed as previously described (1) from the conversion of [^{32}P] GTP to cGMP. Reaction mixtures routinely contained at final concentration 50 mM Tris (pH 7.6) 10 mM theophyline, 2.7 mM cGMP, 4 m M $MnCl_2$, 15 mM creatine phosphate, 0.5 mg/ml creatine kinase (155 U/mg), and 1 mM [α^{32}P] GTP (2×10^6 dpm). Reactions were initiated by addition of 25 μl of enzyme sample to 50 μl of reaction mixture and conducted for 7 min at 37°C. Assays were terminated by addition of 150 μl of 1 N $HClO_4$. Cyclic GMP was isolated by chromatography on Dowex 50 and alumina as described by White and Karr (33).

Assay of TXB_2. Ex vivo production of TXA_2 by incubates of isolated glomeruli from diabetic and control rats was determined by radioimmunoassay of media TXB_2, a stable metabolic end product of TXA_2. Reagents for the TXB_2 assay were purchased from Dupont New England Nuclear. The assay has been previously reported in detail by our laboratory (34–36).

Statistics. Except where specifically indicated in the Results, experiments were routinely performed in triplicate. The statistical significance of differences between mean values was determined by ANOVA followed by the Fisher multiple comparison method.

Results

Blood glucose was elevated in 1–2-wk (428±20 mg/dl) and 2-mo (469±12 mg/dl) diabetic rats (D) compared with values in age-matched controls (C), but values were not significantly different between the two diabetic groups. Blood glucose values in the insulin-treated diabetic groups were only modestly increased above nondiabetic levels (1–2-wk C, 105±5 mg/dl; D, 123±8; 2-mo C, 103±6; 2-mo D, 138±10). Body weight was similar in 1–2-wk D (293±7 g) and age-matched C (285±6 g), but was reduced in 2-mo D (275±14 g) compared with age-matched C (389±5 g). Body weight of 1–2-wk and 2-mo D treated with insulin did not differ from corresponding values for age-matched C.

Fig. 1 compares basal cGMP and cGMP responses to a maximal stimulatory concentration of CCh or the Ca^{2+} ionophore A23187 in glomerular incubates from 1–2-wk or 2-mo D to those of glomeruli from age-matched C. Basal cGMP generation and both the absolute (Fig. 1) and relative (C, 2.6±0.2-fold increase over corresponding basal; D, 1.9±0.2-fold increase, $P < 0.05$) cGMP responses to CCh were significantly reduced in 1–2-wk D compared with C. By contrast, the cGMP responses to A23187 did not differ significantly in glomeruli from C compared to values in glomeruli from 1–2-wk D, although absolute values were modestly lower in glomeruli from D.

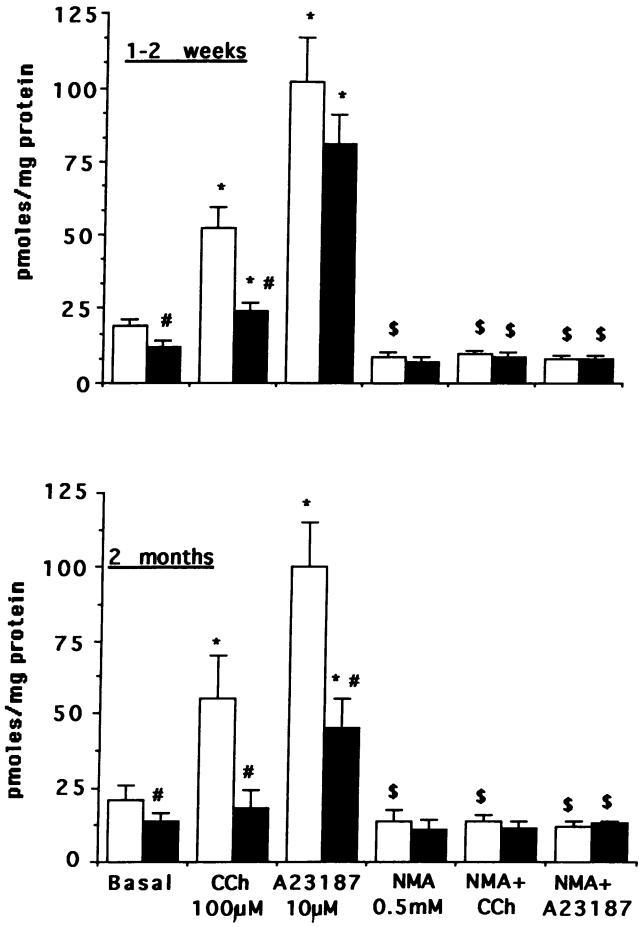

Figure 1. Basal, CCh- and A23187-responsive cGMP in glomeruli from diabetic and control rats. Glomeruli were isolated from 1–2-wk or 2-mo diabetic (■) and age-matched control rats (□) and initially incubated for 30 min at 37°C in KRBG in the presence or absence of 0.5 mM NMA. CCh or A23187 were then added where indicated to give the final concentrations shown and the incubation continued for an additional 15 min. Results shown are means±SE of triplicate determinations from three separate experiments. $^{*}P < 0.05$ vs. basal; $^{\#}P < 0.05$ vs. control; $^{\$}P < 0.05$ vs. no NMA.

At 2 mo after induction of diabetes, basal glomerular cGMP generation remained reduced in D compared with that in age-matched C. In contrast to results obtained with glomeruli from 1–2 wk D, no glomerular cGMP response to CCh was detectable, and the cGMP response to A23187 was significantly impaired in glomeruli from 2-mo D compared with that from age-matched C. N^{G}-monomethyl-L-arginine (NMA), a competitive inhibitor of L-arginine metabolism, has been used to assess cGMP synthesis which is dependent upon endogenous generation of NO via L-arginine metabolism by NO synthetase (37). NMA significantly reduced, by ~ 50%, basal cGMP of glomeruli from 1–2-wk and 2-mo C, but did not significantly alter basal cGMP in glomeruli from D. In the presence of NMA, basal cGMP generation did not differ significantly in glomeruli from C vs. corresponding values from D (Fig. 1). This suggests that a higher basal rate of NO-dependent cGMP generation by glomeruli from C largely accounted for the differences in basal cGMP otherwise seen. As is also shown in Fig. 1, NMA abolished CCh and A23187 induced increases in glomerular cGMP in all glomerular incubates, consistent with the dependence of these responses on endogenous NO production from arginine. Basal cGMP and cGMP responses to CCh and A23187 of glomeruli isolated from 1–2-wk or 2-mo D whose blood glucose had been maintained at near normal levels by insulin infusion did not differ significantly from the corresponding values in glomeruli from nondiabetic rats which are shown in Fig. 1.

Fig. 2 compares the concentration response relationship between CCh and glomerular cGMP in 2-wk D vs. C. The lowest concentration of CCh which detectably increased cGMP in glomeruli from C was 10 μM, with a maximal three-fold increase in cGMP observed in response to 100 μM CCh. By contrast, in glomeruli from 2-wk D, no significant increase in cGMP was observed with 10 μM CCh; a maximal increase in cGMP was observed with 100 μM CCh which was less than two-fold over basal values. In other studies (not shown) addition of 150 U/ml of SOD 15 min before addition of CCh did not alter the concentration response relationship between CCh and cGMP in glomeruli from either C or D compared to those depicted in Fig. 2. As shown in Fig. 3, the cGMP response of glomeruli from D to a maximally effective concentration of CCh (100 μM) was ~ 35–40% of control values when responses were examined at 5, 15, or 45 min after addition of CCh.

Fig. 4 illustrates the effects of maximal stimulatory concentrations of atrial natiuretic peptide (ANP) and NP on cGMP responses of glomerular incubates from 1–2-wk and 2-mo D compared to values in age-matched C. As shown, the cGMP responses to ANP were not significantly different in glomeruli from 1–2-wk or 2-mo D compared to corresponding values in age-matched C. NP-responsive cGMP also did not differ in glomeruli from 1–2-wk D compared to C (Fig. 4). However, the cGMP response to NP was lower in glomeruli from 2-mo D versus values in glomeruli from age-matched C. The magnitude of the glomerular cGMP responses to both ANP and NP greatly exceeded those to CCh (Fig. 1) in both C and D. More-

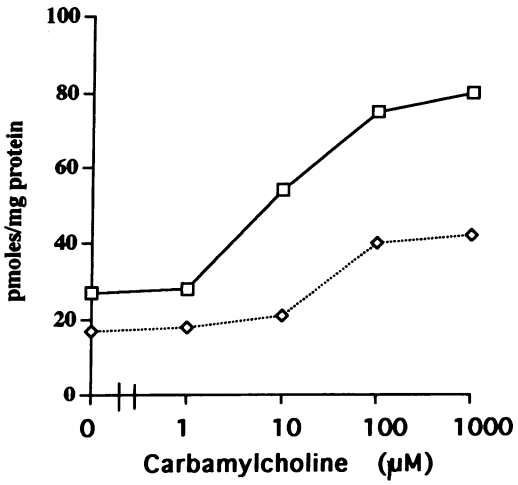

Figure 2. Concentration response relationship between CCh and cGMP in glomeruli isolated from 2-wk diabetic (◇) and control (□) rats. Glomeruli were incubated as described in the legend to Fig. 1. The indicated concentration of CCh was present for the final 15 min of incubation. Results shown are the average of triplicate determinations from a single experiment, verified in a separate study.

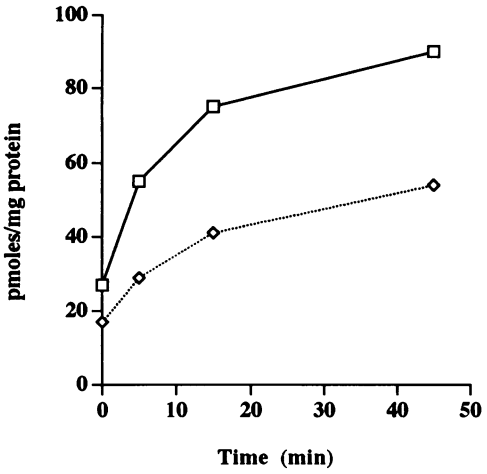

Figure 3. Time course of carbamylcholine action on glomerular cGMP of 2-wk diabetic (◇) and control (□) rats. Incubations were conducted as described in the legend to Fig. 1. CCh (100 μM) was present for the times indicated.

over, in contrast to its effects on CCh and A23187 responsive cGMP (Fig. 1), NMA did not alter cGMP responses to ANP or NP in glomeruli from either C or D (not shown).

Fig. 5 shows the concentration response relationship between NP and cGMP in glomerular incubates from 2-mo D compared to C. Concentrations of NP as low as 1 μM were tested. However, the lowest concentration of NP which significantly increased cGMP content of glomeruli from C (~ 2-fold over corresponding basal) and D (1.4-fold over basal) was 0.1 mM. Maximal 18- and 12-fold increases in cGMP content were observed in response to 10 mM NP in glomeruli from 2-mo C and D, respectively. The absolute increases in cGMP in response to each concentration of NP tested were approximately 50–60% lower in glomerular incubates from 2-mo D compared with C (Fig. 5). Addition of 150 U/ml of SOD 15 min before addition of NP failed to alter the concentration response relationship between NP and cGMP in glomeruli from either C or D compared to those shown in Fig. 5.

Fig. 6 shows the concentration response relationship between NP and guanylate cyclase activity in soluble fractions of homogenates of glomeruli from 2-mo D and C. The lowest concentration of NP tested was 1 μM. A significant 2-fold increase in enzyme activity was observed with 0.1 mM NP in soluble fractions from both D and C, the lowest concentration of NP effective in activating guanylate cyclase in either glomerular preparation. A maximal 12-fold stimulation was obtained in response to 1 mM NP in both preparations, with no significant differences in the concentration response relationship between NP and soluble guanylate cyclase activity observed in fractions of glomeruli from C vs. D.

Fig. 7 shows the effects of addition of 5 mM L-arginine to the incubation media on basal cGMP and cGMP responses to CCh and A23187 in glomerular incubates from 2-mo D. Results are compared to values obtained in glomeruli from C incubated in the presence of L-arginine. As is shown in Fig. 7, addition of L-arginine increased basal cGMP generation by glomeruli from 2-mo D compared to values without added L-arginine. However, addition of L-arginine did not restore a detectable cGMP response to CCh. The absolute increase in cGMP in response to A23187 was also higher in glomeruli from 2-mo

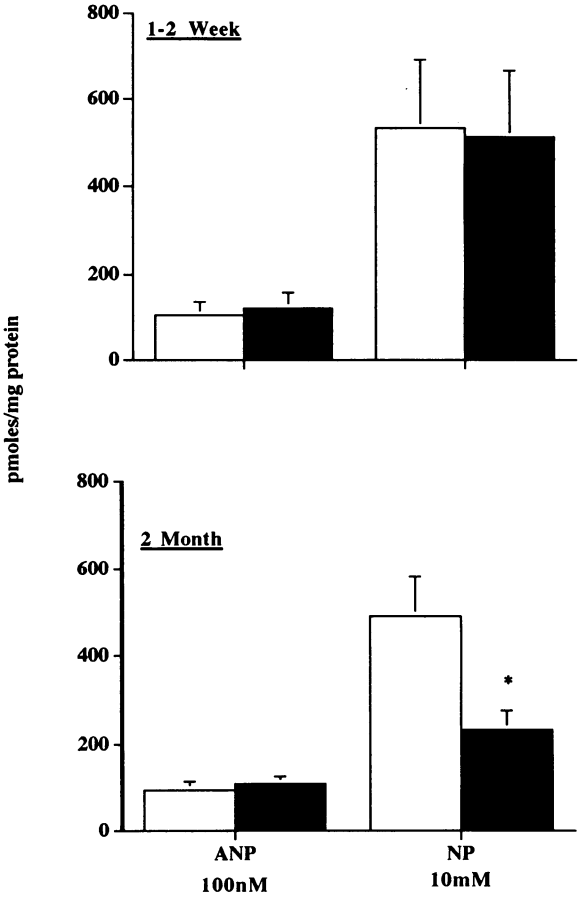

Figure 4. Effects of ANP and NP on cGMP content of glomerular incubates isolated from 1–2-wk and 2-mo diabetic (■) and control (□) rats. Glomeruli were incubated as described in the legend to Figure 1. ANP or NP was added to give the concentration shown for the final 15 min of incubation. Basal values are shown in Fig. 1. Results are means±SE of triplicate determinations from three separate experiments. $*P < 0.05$ comparing diabetic to corresponding control value.

D incubated in 5 mM L-arginine compared with that in glomeruli exposed to A23187 without added L-arginine. However, relative cGMP responses to A23187 expressed as a fold increase over corresponding basal values with and without L-arginine were not altered (without L-arginine, 2.4-fold; with L-ar-

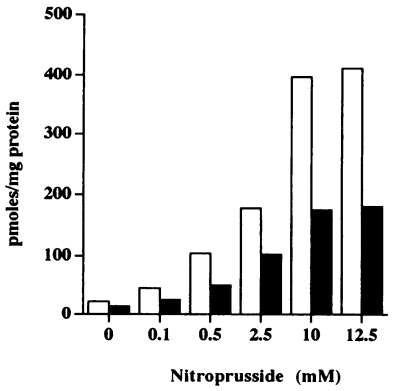

Figure 5. Concentration response relationship between NP and cGMP content of glomeruli from 2-month diabetic (■) and control (□) rats. Glomeruli were incubated as described in the legend to Fig. 1. NP was added to give the final concentration indicated during the final 15 min of incubation. Results shown are the average of triplicate determinations from a single experiment, verified in a separate study.

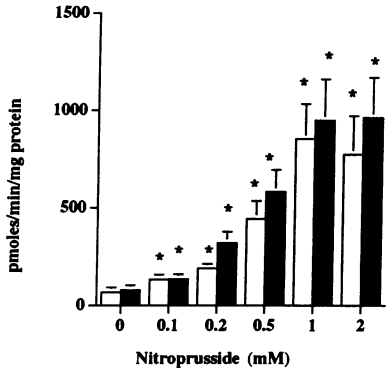

Figure 6. Concentration response relationship between NP and soluble guanylate cyclase activity in glomeruli from 2-mo diabetic (■) and age-matched control rats (□). Guanylate cyclase activity was determined in the 100,000*g* soluble fractions of glomerular homogenates from 2-mo diabetic and control rats. NP was present where indicated at the final concentration shown. Results are means±SE of triplicate determinations from three separate experiments. $^{*}P < 0.05$ vs. value obtained in the absence of NP.

ginine, 2.4-fold over the corresponding basal values). As is also shown in Fig. 7, although addition of 5 mM L-arginine to the incubation medium increased basal cGMP and the absolute increase in cGMP induced by A23187 in glomerular incubates from 2-mo D, these values remained significantly lower than corresponding values obtained in glomeruli from C incubated with L-arginine. In the studies conducted in Fig. 7, glomeruli were preincubated with added L-arginine for 30 min before addition of CCh or A23187. The incubation was then continued for an additional 15 min. In other studies (not shown) glomeruli were incubated with L-arginine for 5 min or 2 h before addition of CCh or A23187. Analogous to results shown in Fig. 7, exposure to L-arginine for 5 min or 2 h did not restore the cGMP response to CCh in glomeruli from 2-mo D and did not alter the relative differences in basal, or A23187-responsive cGMP observed in glomeruli from 2-mo D compared to corresponding control values.

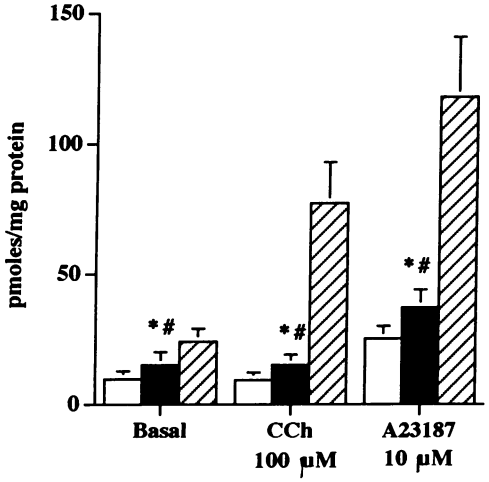

Figure 7. Effects of L-arginine on basal cGMP and cGMP responses to CCh and A23187 in glomeruli from 2-mo diabetic and control rats. Glomeruli were isolated from 2-mo diabetic (□, ■) or age-matched control (▨) rats and incubated for 30 min in KRBG in the presence (■, ▨) or absence (□) of 5 mM L-arginine. CCh or A23187 was then added where indicated and the incubation continued for 15 min. Results shown are means±SE of triplicate determinations from three separate experiments. $^{*}P < 0.05$ vs. no arginine; $^{\#}P < 0.05$ vs. control.

Fig. 8 illustrates the effects of prior exposure of glomeruli to the PKC inhibitors staurosporine or H-7 on basal cGMP and increases in cGMP induced by CCh in glomeruli from 2-mo D compared to age-matched C. As illustrated in the upper panel of Fig. 8, exposure of glomeruli from C to staurosporine or H-7 had no effects on basal cGMP or increases in cGMP induced by CCh. Similarly, neither staurosporine nor H-7 altered basal cGMP in glomeruli from D. By contrast, exposure of glomeruli from D to staurosporine or H-7 partially restored the cGMP response to CCh. However, both absolute cGMP accumulation in response to CCh in glomeruli from 2-mo D exposed to the

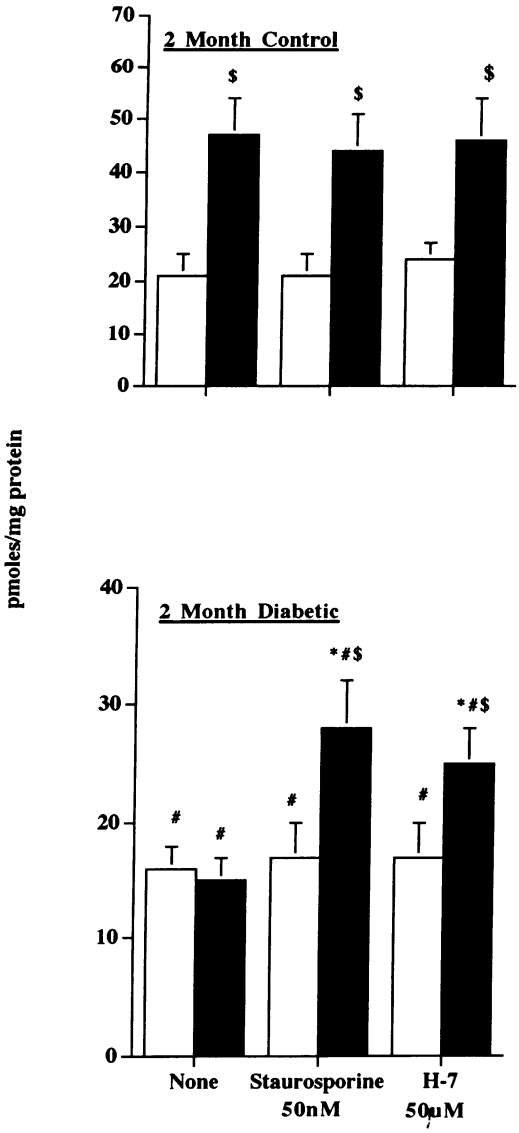

Figure 8. Effects of inhibitors of PKC on basal cGMP and cGMP responses to CCh in glomeruli isolated from diabetic and control rats. Glomeruli were initially incubated in KRBG for 25 min. Where indicated, staurosporine or H-7 was added to give the final concentration shown and the incubation continued for 5 min. Either no further agent (□) or CCh, 100 μM (■) was then added and the incubation continued for 15 min. Results shown are means±SE of triplicates from three separate experiments. $^{*}P < 0.05$ vs. corresponding value obtained in the absence of staurosporine or H-7; $^{\#}P < 0.05$ vs. corresponding value obtained in glomeruli from control; $^{\$}P < 0.05$ vs. corresponding basal.

PKC inhibitors (Fig. 8) and relative increases over the corresponding basal cGMP (C, 2.2-fold increase; D, 1.0; D + staurosporine, 1.6; D + H-7, 1.5-fold) remained reduced compared to corresponding values in glomeruli from age-matched C. The PKC inhibitors had no effect on the cGMP responses to 10 μM A23187 and did not alter responsiveness of glomeruli from 2-mo D to NP when tested at a maximum concentration of NP (10 mM; not shown).

Fig. 9 compares PKC activity in glomeruli from D and C, as assessed by in situ phosphorylation of an 80,000-M_r substrate for PKC which is a member of the MARCKS protein family. Phosphorylation of the 80,000-M_r substrate was approximately 2-fold higher in glomeruli from D compared to C, a finding consistent with activation of glomerular PKC in the D. This conclusion is also supported by significant translocation of PKC from the cystolic to the membraneous cell fractions of glomeruli from D, as previously reported (21). Specific activity of [^{32}P]ATP, as determined by the method of Hawkins et al. (32) after glomerular labeling with [$^{32}P_i$], did not differ significantly in the two glomerular preparations, and thus did not account for the enhanced [^{32}P] incorporation into the 80,000-M_r substrate in glomeruli from D. As is also shown in Fig. 9, H-7 and staurosporine, when tested at those concentrations which restored a cGMP response to CCh in glomeruli from D (Fig. 8), each markedly suppressed phosphorylation of the 80,000-M_r substrate of PKC in both glomerular preparations.

Fig. 10 shows the effects on basal and CCh-responsive glomerular cGMP of Bay U3405, a potent TXA_2/endoperoxide receptor antagonist (34). Bay U3405 did not alter basal cGMP in glomeruli from either 2-mo C or D, or the cGMP response to CCh in glomeruli from C. However, incubation with Bay U3405 clearly restored a cGMP response to CCh in glomeruli from D, although absolute glomerular cGMP responses to CCh remained below corresponding CCh values of C. Consistent with earlier observations (35, 36), ex vivo production of TX by glomeruli from D was significantly higher than that of glomeruli from C as reflected by media TXB_2 accumulation (C, 2.1±0.2 ng TXB_2/mg protein per 45 min; D, 4.3±0.5; $P < 0.05$; mean±SE of determinations from media of those 45-min incubations shown in Fig. 10 which were conducted in the absence of added test agents).

Fig. 11 shows the effects of the TX/endoperoxide analogue U46619, staurosporine, and H-7 on the state of activation of PKC in glomeruli as assessed by phosphorylation of the 80,000-M_r protein substrate for PKC. As shown, exposure of glomeruli to 1 μM U46619 increased 80,000-M_r substrate phosphorylation in glomeruli from C approximately two-fold; 10 μM U46619 did not further increase 80,000-M_r phosphorylation. Prior exposure of glomeruli to 50 nM staurosporine or

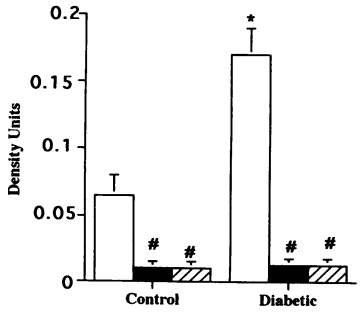

Figure 9. In situ phosphorylation of the 80,000-M_r substrate of PKC in glomeruli isolated from control and diabetic rats in the presence and absence of inhibitors of PKC. After isolation, glomeruli were incubated for 3 h in [^{32}P] orthophosphate as described in the Methods. Where indicated, no further addition (□), H-7 (50 μM) (■), or staurosporine (50 nM) (▨) were present for the entire incubation period. Results shown are means±SE of triplicate determination from three separate experiments. *$P < 0.05$ comparing value in diabetic to corresponding control value. #$P < 0.05$ compared to corresponding basal value.

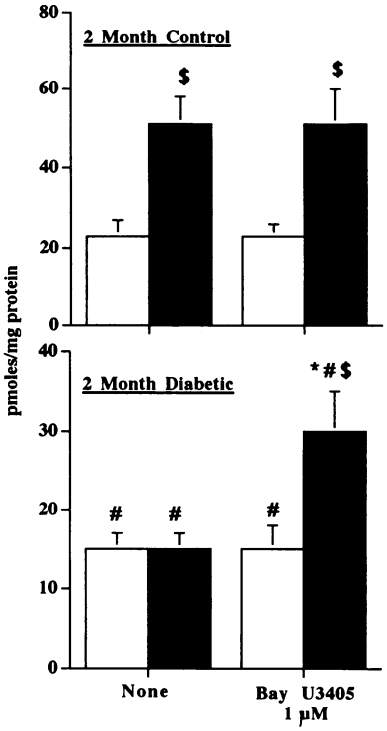

Figure 10. Effects of the TXA_2/endoperoxide receptor antagonist Bay U3405 on basal and CCh-responsive cGMP in glomeruli from control and diabetic rats. Glomeruli were initially incubated for 30 min in KRBG in the presence or absence of 1 μM Bay U3405, as indicated. No further test agent (□) or CCh 100 μM (■) was then added and the incubation continued for 15 min. Results are means±SE of triplicates for three separate experiments. *$P < 0.05$ vs. corresponding value in the absence of Bay U3405; #$P < 0.05$ vs. corresponding value obtained in glomeruli from controls; $$P < 0.05$ vs. corresponding basal.

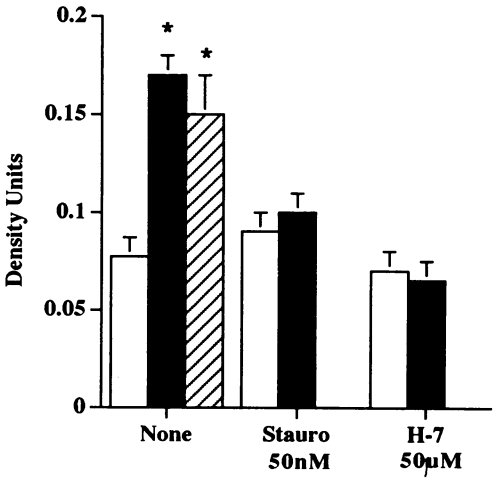

Figure 11. Effects of U46619, staurosporine, and H-7 on 80,000-M_r substrate phosphorylation. Glomeruli were isolated from control rats and incubated for 3 h in [^{32}P] orthophosphate as described in the Methods. Staurosporine and H-7 were present where shown for the final 45 min of incubation. No further agent (□), 1 μM U46619 (■), or 10 μM U46619 (▨) were then added for the final 15 min of incubation. Results shown are means±SE of duplicate determinations from three separate experiments. *$P < 0.05$ compared to corresponding value with no final addition of U46619.

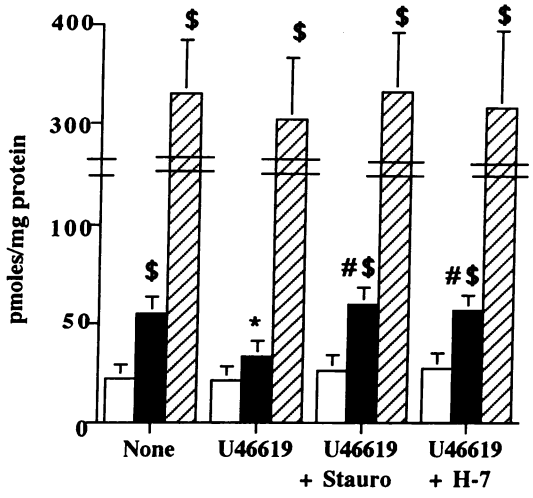

Figure 12. Effects of U46619 on glomerular cGMP responses to CCh and NP in the presence and absence of PKC inhibitors. Glomeruli were isolated from control rats and incubated for 25 min in KRBG. Where indicated, staurosporine, H-7 or U46619 were then added alone or in combination to give the final concentrations shown. The incubation was continued for 5 min. As indicated, no further test agent (□), CCh 100 μM (■), or NP 10 mM (▨) were then added and the incubation continued for 15 min. Results are means±SE of triplicate determinations from three separate experiments. $^{*}P < 0.05$ vs. corresponding value obtained in the absence of U46619. $^{\#}P < 0.05$ vs. corresponding value obtained in the presence of U46619 alone. $^{\$}P < 0.05$ vs. corresponding value with no CCh or A23187.

50 μM H-7 did not detectably alter basal 80,000-M_r substrate phosphorylation, but abolished the increases induced by U46619.

Fig. 12 shows the effects of prior exposure of glomeruli to U46619 on basal cGMP and the cGMP responses to CCh and NP. Prior exposure of glomeruli to 1 μM U46619 blocked the cGMP response to CCh. By contrast, 1 μM U46619 had no effect on basal cGMP or on the cGMP response to NP or to 10 μM A23187 (not shown). As is also shown in Fig. 12, H-7 or staurosporine prevented U46619 suppression of the cGMP response to CCh. By contrast, staurosporine and H-7 had no effect on basal cGMP or the cGMP responses to NP (Fig. 12) or 10 μM A23187 (not shown).

Discussion

The results of the current study demonstrate that NO-dependent cGMP generation is impaired in glomeruli isolated from the streptozotocin diabetic rat. Recently, Wang and co-workers (18) have also reported reductions in the cGMP responses to CCh and NP in glomeruli isolated from this diabetic model. In the present study, alterations were observed as early as 1 wk after induction of diabetes, and were more pronounced after 2 mo of disease. Consistent with the recently reported findings of Wang et al. (18), administration of sufficient insulin to maintain near normal glucose levels in D prevented the alterations in glomerular cGMP generation otherwise observed, and thus implicated the effects of the diabetic state on these glomerular changes rather than those of streptozotocin. Although total cGMP accumulation by glomerular incubates was the end point monitored in the present study, all incubations were conducted in the presence of a potent inhibitor of phosphodiesterase activity. Thus, the alterations in cGMP accumulation observed predominantly reflect alterations in cGMP generation. The latter is determined by the state of activation of at least three distinct forms of guanylate cyclase (20). The cytosolic form of the enzyme contains a heme group and is activated by NO via formation of a nitrosyl heme complex (1). The plasma membrane and cytoskeletal forms of guanylate cyclase are not activated by NO (20). The present results suggest that the changes in cGMP generation observed in glomeruli from D may involve impaired generation of endogenous NO, impaired expression of NO action, or both. Thus, cGMP responses to CCh and A23187, two agents whose actions on cGMP in other cells are expressed through production of endogenous NO via stimulation of the calcium-calmodulin–dependent NO synthetase system (38), were impaired in glomeruli from D. An analogous mechanism of action of these agents in glomeruli is supported by the observation that NMA, a competitive inhibitor of arginine conversion to NO by NO synthetase (37), blocked the increases in cGMP induced by CCh and A23187 in glomeruli from both D and C. NMA also abolished the differences in basal cGMP generation observed in glomeruli from D vs. C, an observation which implied that reduced basal generation of cGMP in glomeruli from D reflected largely a change in the basal rate of NO-dependent cGMP generation in these glomeruli. Conversely, a high concentration of arginine increased absolute basal cGMP generation and absolute cGMP values in the presence of CCh and A23187 in glomeruli from both C and D. Thus, arginine availability may be a determinant of absolute NO generation in isolated glomeruli, as in the isolated perfused kidney (39). However, exogenous arginine failed to abolish the differences in either basal or A23187 responsive cGMP between glomeruli from C and D, and did not restore a cGMP response to CCh in glomeruli isolated from 2-mo D. Accordingly, these changes in glomeruli from D could not be attributed to arginine depletion. A relatively selective impairment of NO responsive cGMP generation in glomeruli from D was supported by studies with ANP, which activates the plasma membrane form of guanylate cyclase through a receptor mediated mechanism that does not involve NO (20). cGMP responses to ANP did not differ in glomeruli from C and D.

The impairment of glomerular cGMP responses to CCh, A23187, and NP was progressive in D over the 2-mo study period. Thus, after 1–2 wk of diabetes basal NO-dependent cGMP generation was reduced, the cGMP response to CCh was blunted, but cGMP responses to A23187 and NP did not differ in glomeruli from D compared to C. After 2 mo of diabetes, a cGMP response to CCh was undetectable and the responses to both A23187 and NP were blunted. The mechanisms responsible for these changes remain to be defined. General possibilities include reduced NO generation, quenching of NO, or an alteration in the soluble guanylate cyclase system per se which impairs its responsiveness to NO. Since soluble guanylate cyclase prepared from glomeruli of C and D responded indistinguishably to NP, a defect in this enzyme per se seems unlikely. We attempted to measure basal and stimulated endogenous NO production of glomeruli from C or D directly from nitrite production, employing the Griess reaction (40). However, consistent with an earlier report (40), nitrite production was not detectable in glomeruli isolated from either C or D and incubated for 1–24 h in the presence or absence of concentrations of CCh or A23187 which gave maximal increases in cGMP. This presumably reflects a low level of NO production

in glomeruli. Accordingly, the possibility of impaired endogenous NO production in glomeruli from D as a basis for reduced basal cGMP generation or the impaired cGMP responses to CCh and A23187 could not be directly excluded.

In the case of the altered CCh responses, impaired NO generation might reflect defects in membrane receptor binding, coupling of the receptor to phospholipase C, the subsequent increase in intracellular Ca^{2+}, or an alteration at the level of Ca^{2+}-calmodulin–dependent NO synthetase (23). The last possibility seems unlikely in glomeruli from 1–2-wk D, since there was no alteration in the cGMP response to A23187, which is also mediated through stimulation of the Ca^{2+}-calmodulin–responsive NO synthetase system (38). Altered NO-synthetase activity is not excluded in glomeruli from 2-mo D, where responses to both CCh and A23187 were reduced. However, findings in glomeruli from 2-mo D imply that at this later stage of diabetes impaired endogenous generation of NO by glomeruli can not alone fully account for the changes in cGMP observed. Thus, in contrast to the finding after 1–2 wk of diabetes, the cGMP response to NP, which serves as an exogenous, nonenzymatic source of NO, was clearly impaired in glomeruli from 2-mo D. This observation and the unaltered direct response of soluble guanylate cyclase to NP in subcellular fractions of glomeruli from 2-mo D compared to those from age-matched C strongly imply a role for increased quenching of NO in intact glomeruli from D. Formation of advanced end products of glycosylation (12), reactive oxygen species (14, 17) lipid peroxides (5), or oxidized lipoproteins (41) all may be increased as a consequence of the diabetic state. Each of these moieties can quench NO (5, 12, 14, 17, 41). A progressive increase in NO quenching might explain the findings in isolated glomeruli from D at both the early and later time points of study. Thus, at 1–2 wk, enhancement of NO quenching may have been sufficient to suppress basal cGMP and the smaller increases in cGMP, and presumably in NO generation, induced by CCh relative to the more marked increases in NO and cGMP induced by A23187 or NP. A greater degree of NO quenching at 2 mo may have abolished the glomerular cGMP response to CCh and suppressed NO-mediated cGMP responses to A23187 and NP. With respect to the possibility that reactive oxygen species may quench NO responses in tissues of diabetics, SOD at a concentration of 150 U/ml or less has been reported to restore NO-mediated relaxation of aorta from diabetic rats (42, 43). In the present study, prior exposure of glomeruli from C or D to 150 U/ml of SOD did not alter the concentration–response relationship between cGMP and either CCh or NP. These limited observations, however, do not definitively preclude a role for superoxide or reactive oxygen species in the suppression of NO mediated increases in cGMP in glomeruli.

The influence of inhibition and activation of PKC on cGMP generation in glomeruli from both C and D implicate this signaling system in the modulation of the cGMP response to cholinergic stimuli in glomeruli. Activation of PKC has been reported to suppress NO-mediated cGMP responses to cholinergic and other stimuli by complex mechanisms. These include increased generation of prostaglandin endoperoxides which may quench NO (5), direct phosphorylation, and inhibition of the constitutive form of NO synthetase (23), or selective inhibition of cholinergic responses by interference with the receptor-mediated signaling pathway proximal to NO synthetase (44). In earlier studies (21), the state of activation of PKC in glomeruli from D had been assessed using the indirect index of subcellular distribution of enzymatic activity. Compared with C, there was significant translocation of PKC from the cytosolic to the membraneous cell fraction of glomeruli from D, a finding consistent with activation of PKC in glomeruli from D (21). In the present study, PKC activity in glomeruli from D and C was assessed from in situ phosphorylation of the MARCKS protein, a specific endogenous substrate of the enzyme. Consistent with our earlier findings (21), this more direct method of assessment of PKC also supported the conclusion that PKC is activated in glomeruli of D compared to C (Fig. 9).

Previous studies have also indicated that PKC is activated in glomeruli from D at least in part by the combined actions of high glucose concentrations (21, 22) and increased glomerular production of TX (34–36, 45), each of which independently increases cellular diacylglycerol production through distinct metabolic pathways (22, 46). Results of the present study are consistent with roles for both enhanced glomerular generation of TX by glomeruli of D and TX-mediated activation of PKC in the suppression of the cGMP response to CCh in glomeruli. Thus, in the present study, TX production was significantly higher in glomeruli from D than in those from C, analogous with earlier findings (35, 36). The TXA_2/endoperoxide antagonist Bay U3405, which has been shown to attenuate the progression of albuminuria and glomerular pathologic changes in D when given by chronic oral administration (36), clearly restored a cGMP response to CCh in glomeruli from D (Fig. 10). Moreover, exposure of glomeruli from nondiabetic rats to the stable TX/endoperoxide analogue U46619 activated PKC, as reflected by enhanced phosphorylation of the 80,000-M_r protein substrate of PKC. U46619 also significantly suppressed the cGMP response to CCh, whereas the cGMP responses to NP and A23187 were not altered. The actions of U46619 to enhance 80,000-M_r phosphorylation and suppress a cGMP response to CCh were both blocked by two structurally distinct inhibitors of PKC, staurosporine and H-7. Since neither of these agents is totally selective with respect to inhibition of PKC, alternative explanations for their actions can not be excluded. Nevertheless, each agent, when tested at a concentration which clearly inhibited PKC activity in glomeruli from both C and D (Fig. 9), partially restored the cGMP response to CCh in glomeruli from 2-mo D, but did not alter basal cGMP or cGMP responses to A23187 or NP. In the studies shown in Fig. 9, the PKC inhibitors were added prior to [$^{32}P_i$], which accounts for the dramatic declines in ^{32}P-incorporation into the 80,000-M_r substrate of PKC in these glomerular incubates. The failure of PKC inhibitors to completely restore the CCh response in glomeruli from D may, conversely, be related to an inability to reverse fully in vitro key PKC-mediated phosphorylation events that had occurred in the glomerulus in vivo. When added before U46619, the PKC inhibitors did completely prevent both 80,000-M_r phosphorylation and suppression of the cGMP response to CCh induced by U46619 in vitro in glomeruli from C. Activation of PKC may specifically suppress the cholinergic signaling pathway in glomeruli proximal to NO synthetase, in that effects of PKC on NO synthetase (23) or NO quenching (47) would be expected to modify basal cGMP and cGMP responses to A23187 or NP. The finding that the PKC inhibitors did not restore the cGMP response to NP in glomeruli from D implied that activation of PKC did not induce quenching of NO in glomeruli from D, and further supported a selective action of activation of PKC on the CCh

response. In this regard, CCh has previously been reported to induce inositol phospholipid turnover (48) and increase cytosolic Ca^{2+} (49) in isolated glomeruli from nondiabetic rats with a concentration response relationship identical to that observed in the present study for CCh stimulation of cGMP. Activation of PKC has been shown to suppress CCh induced inositol phospholipid turnover in other cell systems (44), a site of PKC action which might also explain the present findings. Other mechanisms of PKC actions on NO-dependent cGMP in the glomerulus are not excluded by the present data.

Thus, the current study suggests that diabetes impairs NO-responsive cGMP generation in glomeruli by both PKC-dependent and -independent mechanisms. In the glomerulus, NO is known to be generated endogenously via Ca^{2+}-calmodulin–dependent NO-synthetase by endothelial cells (50) and by epithelial cells of the macula densa (51). An inducible form of NO-synthetase is present in mesangial cells (52). The present study of intact glomeruli does not localize (*a*) the cellular sites of impaired NO generation and/or impaired NO action to increase cGMP induced by diabetes; or (*b*) the cellular sites of PKC activation within the glomerulus. Clearly, the multiple cell types within the glomerulus and the possibility that cGMP generation and PKC are altered differentially in these various cell types by diabetes all preclude a precise delineation of cellular mechanisms involved in modulation of NO-responsive cGMP, and also limit interpretation of the potential pathophysiologic consequences of the changes observed. In this regard, NO production and actions at several sites both within and outside the glomerulus may potentially influence vascular tone and glomerular hemodynamics in diabetes. Enhanced generation of NO has been implicated in the vascular dysfunction (8, 9) and hyperfiltration (10) of early diabetes. Our studies in isolated glomeruli do not support a role for enhanced NO-dependent cGMP generation by this organelle as mediator of hyperfiltration of early diabetes, since basal NO-dependent cGMP generation and the cGMP response to CCh were clearly suppressed by 1–2 wk after initiation of diabetes at a time when hyperfiltration in D is well documented (53). However, because of the heterogeneity of cell types present in the glomerulus, the possibility can not be excluded that while total NO-dependent cGMP generation by this organelle is reduced by diabetes, cGMP generation by a specific cell type within the glomerulus, such as the mesangial cell, might be increased. This, in turn, might contribute to glomerular hyperfiltration. Moreover, increased NO-mediated vasodilation at intrarenal sites outside the glomerulus, such as the afferent arteriole, could also clearly contribute to hyperfiltration of early diabetes.

Despite the limitations of the current model, it is reasonable to suggest that the suppression of NO-dependent cGMP generation in glomeruli of diabetics may be involved in the development of glomerular capillary hypertension, proteinuria and glomerulosclerosis, since these same glomerular changes occur in nondiabetic rats when NO generation is inhibited (7). The adverse consequences of impaired NO-dependent cGMP generation in the glomerulus in diabetes may also be compounded by concurrent increases in glomerular TX production in this disorder (34–36). Studies in the diabetic rat indicate that TX contributes to the initiation and progression of diabetic glomerulopathy (34–36, 54, 55) through its action as a vasoconstrictor (56) and as a stimulus to mesangial matrix production (57, 58). Increases in cellular cGMP can antagonize these actions of TX (59), and conversely TX may antagonize glomerular cGMP generation in response to at least some stimuli, as demonstrated in the present study. Accordingly, an important local counterregulatory mechanism may be altered in the glomeruli of diabetics.

Acknowledgments

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