PRELIMINARY REPORT

Impaired Nitric Oxide Release by Glomeruli From Diabetic Rats

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Basal nitric oxide (NO) production and NO responses to carbamylcholine (CCh) and the Ca^{2+} ionophore A23187 were measured with a NO electrode in glomeruli isolated from 2 to 3-month–diabetic versus age-matched control rats. In the presence of CCh or A23187, NO production was markedly reduced in glomeruli from diabetic versus control rats. Spontaneous generation of NO by *s*-nitrosopenicillamine (SNAP) was also reduced in the presence of glomeruli from diabetic rats as compared with values either in control glomeruli or in buffer alone. The results demonstrate an impairment of NO generation and/or stability in glomeruli from 2 to 3-month–diabetic rats, which correlates with the previously observed suppression of NO-dependent glomerular cyclic guanosine 3′,5′-monophosphate (cGMP) induced by diabetes.

BASAL CYCLIC GUANOSINE 3′,5′-monophosphate (cGMP) generation and increases in cGMP in response to carbamylcholine (CCh), the Ca^{2+} ionophore A23187, and nitroprusside are progressively impaired in glomeruli isolated from diabetic rats as compared with age-matched controls.[1,2] Basal cGMP and increases in cGMP induced by CCh and A23187 were reduced after 1 to 2 weeks of diabetes.[1] After 2 months of diabetes, the glomerular cGMP response to CCh was abolished and that to nitroprusside was significantly impaired.[1] An impairment of nitric oxide (NO)-dependent cGMP may involve reduced NO generation, quenching of NO, or a reduction in soluble guanylate cyclase activity. In our previous study, soluble guanylate cyclase activity and enzyme responses to nitroprusside were not different in cytosolic fractions of glomeruli from control and diabetic rats,[1] suggesting reduced NO generation, stability, or both as an explanation for the impaired NO-dependent cGMP generation in glomeruli from diabetic rats. However, basal and CCh- or A23187-responsive NO productions were not measurable from NO_2 production by the Griess reaction in glomeruli from either control or diabetic rats. In the present study, we compared NO production in glomeruli from 2 to 3-month–diabetic and age-matched control rats basally and in response to CCh and A23187 using a NO electrode. NO generation in response to *s*-nitrosopenicillamine (SNAP), an agent that generates NO spontaneously in aqueous solutions,[3] was also assessed as an index of NO stability in the glomerular preparations. The results provide the first direct evidence that impairment of NO-dependent cGMP in glomeruli from diabetic rats is due to an impairment of NO generation, stability, or both.

MATERIALS AND METHODS

Female Sprague-Dawley rats (180 to 200 g, Zivic-Miller Laboratories, Pittsburgh, PA) were age- and weight-matched and placed in one of two groups: group I, 2- to 3-month–diabetic rats, and group II, age-matched controls. Diabetic rats received 60 mg/kg streptozotocin in sterile 0.010-mol/L citric acid in saline (pH 4) by tail vein. Blood glucose was determined on tail-vein blood with a blood glucometer (Diascan-S, Home Diagnostics, Eatontown, NJ) 3 days after injection of streptozotocin. Only diabetic rats with blood glucose levels greater than 300 mg/dL 3 days after administration of streptozotocin were included in the study.

Isolation of Glomeruli

Glomeruli were isolated from the kidneys of three rats for each experiment, as previously described.[1] Glomeruli were washed and suspended in 3 mL Krebs buffer containing 10 mmol/L HEPES (pH 7.4), 5 mmol/L glucose, and 8 U/mL superoxide dismutase. Glomerular suspensions were maintained at 37°C in a CO_2 incubator (5% CO_2, 95% room air) before making measurements.

Measurements of NO

NO levels were measured at room temperature using a commercially available NO electrode and meter (Iso-NO) obtained from World Precision Instruments (Sarasota, FL). The design of the electrode has been described in detail previously.[4,5] The Iso-NO has been used to measure NO production by cultured endothelial and vascular smooth muscle cells.[6-8]

The electrode was calibrated daily by titrating a solution of 0.1 mol/L H_2SO_4, 0.14 mol/L K_2SO_4, and 0.1 mol/L KI with KNO_2 (36 to 360 nmol/L) in an atmosphere of N_2. Under these conditions, KNO_2 is quantitatively converted to NO. NO diffuses through a gas-permeable membrane and is oxidized at a platinum electrode. The current created is proportional to the rate of diffusion of NO through the membrane and to the concentration of NO in the solution bathing the membrane. As previously reported,[6] sensitivity of the electrode varied on a day-to-day basis between 2.5 and 4.6 nmol/L/pA. Accordingly, the electrode was calibrated daily. A linear relationship was always obtained between the amperage and NO over a range of concentrations of NO (36 to 360 nmol/L) that encompasses those generated experimentally.

Measurements of glomerular NO release were made in a well of a 12-well tissue culture plate. Each well contained 1 mL (1.5 to 2.5 mg protein) glomerular suspension. The electrode was inserted

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vertically into the well and positioned at the surface of the settled glomeruli. Stable baseline values were obtained for at least 30 minutes before stimulation. NO release was calculated from the amperage generated by comparison with the standard curve. Protein content of each well was determined using a bicinchoninic acid protein assay kit (Sigma Chemical, St Louis, MO). NO release in the presence of ionophore A23187 was linear with protein concentration (1.5 to 3.5 mg) in glomeruli from control and diabetic rats. Results are reported as maximal media concentrations of NO detected after addition of the test agents.

Statistics

Results are the mean ± SEM of determinations from six control and six diabetic glomerular preparations. Significance of differences between mean values was determined by Student's *t* test for unpaired values.

RESULTS AND DISCUSSION

Basal values for NO were not detectable in glomeruli from control or diabetic rats. However, NO was detectable after stimulation of glomeruli from control rats with CCh or A23187. A representative response to CCh and to A23187 in glomeruli isolated from control rats is shown in Fig. 1. Media NO increased rapidly and remained elevated for several minutes in response to either agonist. Increases in NO in response to CCh or A23187 were reduced 70% to 80% by a 30-minute prior exposure of glomeruli to 0.5 mmol/L N^G-monomethyl arginine (NMA).

In the glomerulus, NO is generated via Ca^{2+}-calmodulin–dependent constitutive NO synthetase systems in endothelial cells[9] and epithelial cells of the macula densa.[10] An inducible form of NO synthetase is present in mesangial cells.[11] The present study does not localize the cellular site of NO generation in the glomerulus. However, observations that NO production was increased by agents that mobilize Ca^{2+} and that NMA reduced CCh- and A23187-induced increases in NO accumulation strongly suggest NO generation by constitutive NO synthetase located in endothelial cells or macula densa epithelium.

Previously, we had shown that the cGMP response to CCh was abolished and that the A23187 response was reduced in glomeruli isolated from 2-month–diabetic compared with control rats. Table 1 compares the NO response to CCh and A23187 of glomeruli isolated from diabetic rats versus control rats. NO in glomeruli from diabetic rats was markedly reduced compared with that in control glomeruli in the presence of either CCh or A23187. A NO response to CCh was detected in only one of the six glomerular preparations from diabetic rats. All glomerular preparations from diabetic rats demonstrated a detectable but reduced NO response to A23187 as compared with control glomeruli. Differences between the NO response to A23187 in glomeruli from control and diabetic rats were directionally analogous whether expressed as the maximum concentration of NO detected in the media (Table 1) or as total NO released after addition of test agents (not shown). These results provide direct evidence that the failure of glomeruli from diabetic rats to respond to CCh with an increase in cGMP generation and the reduced cGMP response to A23187 in glomeruli from diabetic rats[1] is due to attenuated NO generation and/or impaired NO stability after stimulation with these agents. In other experiments (not shown), addition of 10 mmol/L arginine did not increase basal NO production to detectable levels and did not restore NO release by glomeruli from diabetic rats to levels observed in glomeruli from nondiabetic rats in the presence of CCh or A23187. Thus, consistent with results obtained in our previous study of NO-dependent cGMP generation,[1] reduced availability of arginine did not account for the impairment of NO release in glomeruli from diabetic rats.

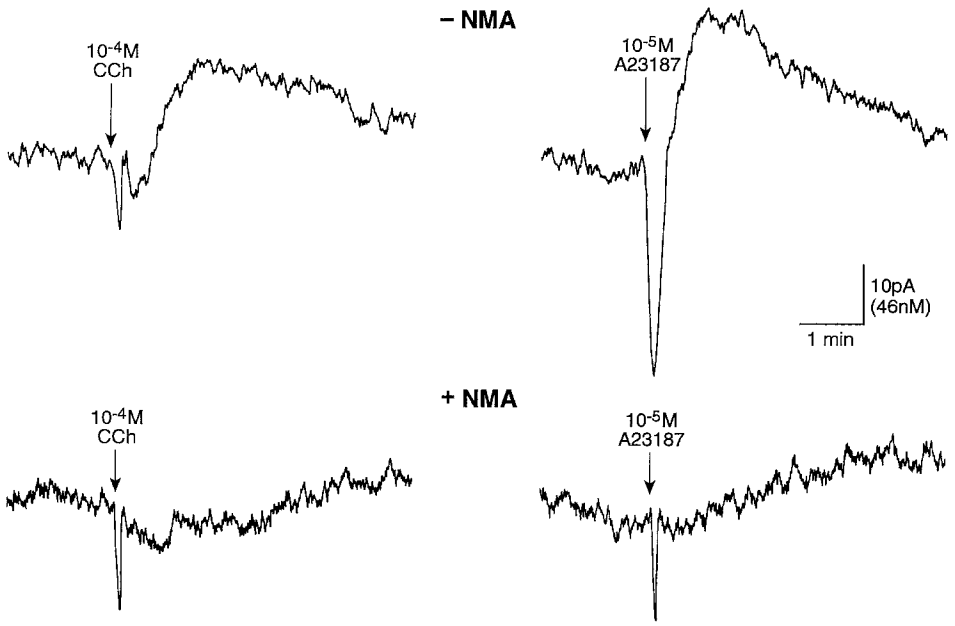

Fig 1. Representative responses of glomeruli from control rats to CCh and A23187. Recorder tracing shows meter output (pA) *v* time (min). Where indicated by arrows, CCh or A23187 were added to glomeruli from control rats.

Table 1. NO Accumulation in Response to CCh, A23187, and Exogenous SNAP in Glomeruli From Control and Diabetic Rats

	CCh (100 μmol/L)	A23187 (10 μmol/L)	SNAP (500 μmol/L)
Control	105 ± 10	155 ± 7	240 ± 10
Diabetic	20	51 ± 8*	148 ± 25*

NOTE. Results are maximal media NO concentrations normalized to 2 mg glomerular protein. Except for the CCh response in diabetic glomeruli, values are the mean ± SE of determinations from 6 control and 6 diabetic glomerular preparations. In 5 of 6 glomerular preparations from diabetic rats, a NO response to CCh was not detectable. The single detectable response is shown. Values for NO generated from SNAP in Krebs buffer alone did not differ significantly from those observed in the presence of control glomeruli.

*$P < .05$ *v* control glomeruli.

Previous studies suggested that the reduction in the cGMP response to CCh and A23187 in glomeruli from diabetic rats was at least in part due to decreased stability of NO.[1] Thus, the cGMP response to nitroprusside was reduced in intact glomeruli from diabetic rats. By contrast, basal and nitroprusside-responsive soluble guanylate cyclase activities of cytosolic fractions of these two glomerular preparations did not differ. In the present study, the stability of NO was measured directly from increases in media NO concentrations induced by SNAP in the presence of glomeruli from control versus diabetic rats. Results of these studies were compared with NO release from SNAP observed in Krebs buffer alone. NO release from SNAP (500 μmol/L) did not differ in the presence of glomeruli from control rats as compared with that in Krebs buffer alone (not shown). However, NO release from SNAP was significantly reduced in the presence of glomeruli from diabetic rats as compared with values from either control glomerular preparations or with values obtained in Krebs buffer (Table 1). This difference pertained whether results were expressed as peak media NO concentration detected (Table 1) or as total NO released (not shown). Since superoxide dismutase was present in the incubation mixture, reduced NO production in the presence of glomeruli from diabetic rats was not attributable to quenching due to O^{2-}. The latter observation is consistent with our previous studies, which demonstrated that superoxide dismutase did not ameliorate the impaired NO-dependent cGMP generation in glomeruli from diabetic rats.[1] The observed reduction in SNAP-induced NO accumulation strongly suggests reduced stability of NO in the presence of glomeruli from diabetic rats. The greater instability of NO likely contributes to the reduced NO response to CCh and A23187 in glomeruli from diabetic as compared with control rats and to the previously reported impairment in NO-dependent cGMP generation. The factors that contribute to NO instability in glomerular preparations from diabetic rats remain to be determined. Several mechanisms, including NO quenching by advanced end products of glycosylation, oxidized lipoproteins, or reactive oxygen species generated during enhanced glucose metabolism, and increased arachidonate oxidation, have been suggested.[12-15] The present detection system for NO should permit direct and systematic in vitro examinations of those factors, including high ambient glucose concentrations, that have been postulated to mediate increased NO quenching in the glomerulus and other cells in diabetes.

Impaired NO production and/or stability in the glomerulus in diabetes and the consequent reduction in cGMP generation may alter several glomerular functions. Recent studies in our laboratory have implicated cGMP generation by glomerular mesangial cells as a negative signal to matrix protein synthesis.[16] Consequently, depressed glomerular cGMP generation may contribute to increased synthesis and accumulation of these proteins. This change, in turn, may lead to an expansion of the mesangial matrix, a characteristic histologic feature of diabetic glomerulopathy that has been linked to a progressive decline in renal function in this disorder.

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