High concentration of glucose inhibits glomerular endothelial eNOS through a PKC mechanism

Shaoyou Chu¹ and H. Glenn Bohlen²

¹Department of Cell Biology and Genetics, University of North Texas Health Science Center, Fort Worth, Texas 76107; and
²Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana 46202

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Chu, Shaoyou, and H. Glenn Bohlen. High concentration of glucose inhibits glomerular endothelial eNOS through a PKC mechanism. Am J Physiol Renal Physiol 287: F384–F392, 2004. First published May 12, 2004; 10.1152/ajprenal.00006.2004.—Kidney glomeruli are important targets of diabetic nephropathy. We hypothesized that a high concentration of glucose could suppress glomerular endothelial nitric oxide synthase (eNOS) by a protein kinase C (PKC) mechanism, as has been found in other tissues. Mouse kidney slices (150–200 μm) were bathed in Hanks’ solution with 100 μM L-arginine and exposed to either 5 or 20–30 mM D-glucose. Immuno-fluorescence identified only eNOS in normal mouse glomeruli. Measurements of glomerular NO concentration with NO-sensitive fluorescent dye (4,5-diaminofluorescein diacetate) using confocal microscopy and NO-sensitive microelectrodes verified that resting glomeruli had active production of NO that was inhibited by Nω-nitro-L-arginine methyl ester. High-concentration (20–30 mM) D-glucose inhibited 60–70% of the NO production within 15–30 min; L-glucose at the same concentration did not have any effect. Inhibition of PKC-β with 100 nM ruboxistaurin prevented eNOS suppression in high-glucose media. Activation of PKC with 100 nM phorbol ester also suppressed the glomerular NO concentration. We concluded that eNOS in the renal glomerular capillary endothelial cells is suppressed by activity of PKC at high-glucose concentrations comparable to those in diabetic animals and humans. The consequence is a rapid decline in the generation of NO in the glomerular endothelial cells in the presence of a high concentration of glucose.

microelectrode; hyperglycemia; confocal imaging; protein kinase C; endothelial nitric oxide synthase

DIABETIC NEPHROPATHY CONTRIBUTES to more than 40% of terminal kidney diseases and is a major diabetic complication (43). Nephropathy develops in both type I and II diabetic patients and in both groups, one of the first indications of abnormal glomerular function is increased filtration of albumin (15). This leakage predicts damage of the glomerular membrane allowing unusually large molecules to sieve between the cells. However, what mechanisms mediate diabetic glomerular pathogenesis is unclear. As the first component of the filtration process begins with glomerular endothelial cells and diabetes impairs endothelial nitric oxide synthase (eNOS) function throughout the body, Jover and Mimran (25) suggested a possible link of impaired endothelial-generated nitric oxide and glomerular permeability function may exist. Although diabetes is associated with chronic eNOS inhibition by a variety of mechanisms (22, 26–28, 39–42), it is not clear if hyperglycemia acutely affects eNOS function in glomeruli in normal conditions or very early stages of diabetic mellitus. We are particularly interested in rapid effects of hyperglycemia because most diabetic humans are only occasionally hyperglycemic. In this context, in vivo microcirculatory studies of the brain (36), skeletal muscle (31), and small intestine (5, 7) in normal rats indicate a rapid, within 45 min, suppression of NO formation. Bioassay studies of endothelial-mediated dilation have also found a rapid loss of NO function in the vasculature of normal humans during acute hyperglycemia (≥300 mg/dl) (2, 48). A common factor in eNOS suppression during acute hyperglycemia is activation of protein kinase C (PKC), and PKC activation is a known factor in diabetic nephropathy (12, 14, 20, 24, 30). The PKC-βII isoform of endothelial cells (2, 4, 7) appears to be particularly important, as judged by the protection offered by blockade of this PKC isoform by ruboxistaurin (formerly LY-333531, Eli Lilly). As the primary goal of the current study, we tested the general hypothesis that acute exposure to a high concentration of glucose (high glucose) inhibits glomerular NO production through PKC suppression of eNOS.

Histological studies have shown that normal glomerular endothelial cells have high expression of eNOS in humans (1, 18) and in mice, the species of this study (47). Functional studies predict that eNOS has an important role in renal protection by maintaining normal glomerular function through inhibition of thrombosis, leukocyte adhesion/activation, apoptosis, and oxidative stress in glomeruli (21, 38). The consequences of both inhibition of eNOS in normal mice and genetic knockout of eNOS in mice are increased vulnerability to experimentally induced glomerulonephritis (21). As hyperglycemia has the ability to rapidly and severely compromise eNOS function through a PKC mechanism in other vascular beds, determining if glomerular capillary NO production is impaired rapidly by high glucose was the major goal of this study. The study of glomerular capillaries in terms of their ability to produce NO would be exceedingly difficult in an in vivo environment. Therefore, thin (150–200 μm) slices of mouse kidney were used as an in vitro model. Using this model, we tested three hypotheses. First, that glomerular NO should be primarily produced by eNOS during normal conditions. This hypothesis was evaluated with immunofluorescence and confocal microscopy to verify the normal glomerular cells predominately expressed eNOS but not neuronal NOS (nNOS) and inducible NOS (iNOS). The second hypothesis was glomerular capillary endothelial cells in the tissue slice conditions used were capable of making NO from eNOS and would respond to bradykinin to increase NO production. We tested this hypothesis by direct measurement of the NO concentration.
with <10-μm outer diameter NO-sensitive microelectrodes and 4,5-diaminofluorescein diacetate (DAF-2 DA) dye to monitor intracellular formation of NO in glomeruli. Third, we hypothesized that hyperglycemia inhibition of eNOS activity would be through PKC mechanism, most likely PKC-β. This hypothesis was tested using DAF-2 DA dye with confocal microscopy on renal slices by demonstration that activation of PKC lowered NO formation and that blockade of PKC-β before a high-glucose exposure protected NO formation.

MATERIALS AND METHODS

Animals. Male ICR mice of 25–30 g body wt were used for the study (Harlan Industries, Indianapolis, IN). The experimental protocol was approved by the Institutional Animal Care and Use Committee of both the University of North Texas Health Science Center and Indiana University School of Medicine. Mice were anesthetized with an intraperitoneal injection of Inactin, a thiobarbiturate (100 mg/kg body wt). The abdominal aorta was cannulated to allow saline perfusion of the kidney to expel the blood, particularly red blood cells. The inferior vena cava was ligated above and below the renal veins and was cut open to allow egress of perfusion fluids. The kidney was perfused with 150 ml cold isotonie physiological saline (Ringer buffer) containing in mM: 130 NaCl, 5 KCl, 2 CaCl2, 1 MgSO4, 1 NaH2PO4, 20 HEPES, and 5.5 d-glucose, pH 7.4) for 5 min to remove all the red blood cells in the kidneys. The kidneys were removed and sliced with a vibrotome (World Precision Instruments, Sarasota, FL) into 150- to 200-μm sections. The tissue sections were immersed in ice-cold Ringer buffer until used for either confocal imaging or microelectrode NO measurements. Each renal slice mounted in a perfusion chamber on the microscope stage was superfused with nitrogen-equilibrated saline and generated an additional 1–2 pA/1,000 nM NO, which was equal to an increase in output voltage of 100–200 or greater mV/1,000 nM from the electrometer. The overall system can resolve 5- to 10-nM changes in [NO], which was sensitive enough for our experimental purpose.

During tissue measurements, the microelectrode tip was placed ~200 μm above the tissue surface to obtain a "0" [NO]. However, we could not detect NO in the bath until the microelectrode tip was placed within ~50 μm above the tissue surface. In the example record shown in Fig. 1, the microelectrode first touched the glomerulus and then was slightly withdrawn to demonstrate that the NO concentration did change with proximity to the tissue. The vast majority of measurements were made by simply pressing the microelectrode tip against the center of the glomerulus. When the sharpened microelectrode tip touched the tissue surface, there was occasionally a brief mechanical artifact that rapidly dissipated. Thereafter, the measurements were quite stable. After the micropipette was completely withdrawn from the tissue into the bath, the current returned to essentially the baseline 0-nM current equivalent. To account for possible electronic drift of the microelectrode, the pre- and postmeasurement current in the bath was used to predict the virtual 0-nM baseline for any time point.

To minimize disturbances during recordings, various preheated solutions containing drugs were slowly added and simultaneously removed from the test chamber. This avoided thermal effects of the solutions and movement of the tissue slice relative to the micropipette. The total volume of the tissue bath was ~15 ml, and the fluid flow was a linear relationship of microelectrode current to NO concentration ([NO]). Microelectrodes had a background current of 3–5 pA in nitrogen-equilibrated saline and generated an additional 1–2 pA/1,000 nM NO, which was equal to an increase in output voltage of 100–200 or greater mV/1,000 nM from the electrometer. The overall system can resolve 5- to 10-nM changes in [NO], which was sensitive enough for our experimental purpose.

Fig. 1. Representative data of nitric oxide (NO)-sensitive microelectrode measurements of NO concentration ([NO]) on renal glomerulus. A: microelectrode contacting the glomerulus followed by a slight withdrawal to reach a steady state. Thereafter, the slice was exposed to 1 mM Nω-nitro-L-arginine methyl ester (L-NAME) and the measured [NO] gradually declined toward the baseline current. B: [NO] was first measured at normal conditions containing 5 mM d-glucose and then the bath was changed to contain 30 mM d-glucose. The delay in the fall of [NO] with glucose represents the slow change of the bathing media followed thereafter by a decline in NO production. A and B: transient spike in the NO current at the end of each record represented withdrawal of the microelectrode to a point ~200 μm above the tissue slice.
3–4 ml/min. The microelectrodes were completely insensitive to the very slow motion of fluid flowing through the test chamber because stopping the flow momentarily had no effect on the recorded electrical current.

**Immunofluorescent staining.** To fix the kidney for immunofluorescent staining, the kidney was first perfused with the PBS to flush out the blood and then freshly prepared 2% paraformaldehyde in PBS for 10 min. The kidney was excised and immersed in the same fixative for 2 h at 4°C. The kidney was then rinsed with PBS and cryoprotected in 30% sucrose in PBS at 4°C for 24 h. Sections of 16-μm thick were cut with a cryostat at −20°C, mounted on glass slides, and immediately processed for immunofluorescent localization of NOS expression according to our previous published methods (11). Briefly, sections were rinsed 5 min with PBS, incubated in washing buffer (PBS containing 50 mM NH4Cl) for 2 × 10 min, and in blocking buffer (washing buffer with 2% BSA and 0.05% Saponin) for 20 min. Sections were incubated with respective primary antibody (5 μg/ml) or preimmune rabbit serum (1:100) in the blocking buffer overnight at 4°C. Primary antibodies used in these experiments are polyclonal antibodies developed in rabbits against eNOS (Sigma, St. Louis, MO; cat. no. N-2643), nNOS (Zymed, San Francisco, CA; cat. no. 61–7000), and iNOS (Transduction Labs, Lexington, KY; cat. no. N32030), respectively. After 4 × 5 min washing, sections were incubated in Alexa488 labeled goat anti-rabbit IgG (5 μg/ml; Molecular Probes, Eugene, OR) for 1 h at room temperature followed by 4 × 5 min washing including a 5-min treatment with 2 μg/ml propidium iodide (Molecular Probes) for nucleus staining. Sections were mounted with ProLong antifade medium (Molecular Probes) and exposed to different treatments. Calcein/AM (2 μM, non-ion-specific dye, Molecular Probes) was used as a dye-loading control for DAF-2 DA following the same experimental procedure. The addition of 0.5 mM L-NAME (NOS inhibitor) inhibited the NO production from the glomerulus in as short as 10–15 min, but 30 min of exposure was more reliable. Exposure of glo-

**Immunofluorescent staining.** To fix the kidney for immunofluorescent staining, the kidney was first perfused with the PBS to flush out the blood and then freshly prepared 2% paraformaldehyde in PBS for 10 min. The kidney was excised and immersed in the same fixative for 2 h at 4°C. The kidney was then rinsed with PBS and cryoprotected in 30% sucrose in PBS at 4°C for 24 h. Sections of 16-μm thick were cut with a cryostat at −20°C, mounted on glass slides, and immediately processed for immunofluorescent localization of NOS expression according to our previous published methods (11). Briefly, sections were rinsed 5 min with PBS, incubated in washing buffer (PBS containing 50 mM NH4Cl) for 2 × 10 min, and in blocking buffer (washing buffer with 2% BSA and 0.05% Saponin) for 20 min. Sections were incubated with respective primary antibody (5 μg/ml) or preimmune rabbit serum (1:100) in the blocking buffer overnight at 4°C. Primary antibodies used in these experiments are polyclonal antibodies developed in rabbits against eNOS (Sigma, St. Louis, MO; cat. no. N-2643), nNOS (Zymed, San Francisco, CA; cat. no. 61–7000), and iNOS (Transduction Labs, Lexington, KY; cat. no. N32030), respectively. After 4 × 5 min washing, sections were incubated in Alexa488 labeled goat anti-rabbit IgG (5 μg/ml; Molecular Probes, Eugene, OR) for 1 h at room temperature followed by 4 × 5 min washing including a 5-min treatment with 2 μg/ml propidium iodide (Molecular Probes) for nucleus staining. Sections were mounted with ProLong antifade medium (Molecular Probes) and imaged with Zeiss LSM510 confocal microscope. Sections were excited with a 488-nm Arg laser and emissions were detected at 505–530 nm (for Alexa488) and 620–680 nm (for propidium iodide) with two separate detectors. Laser power, pinhole size, and detector gain were the same for all samples. 

**Confocal imaging of fluorescent dye-loaded renal slice.** To test the effect of different treatments on glomerular NO production, we preincubated renal slices (150–200 μm) from the same kidney with one of the following solutions for 30 min at 25°C. All solutions were based on the HBSS with 100 μM L-arginine but contained, respectively, 1) 5 mM D-glucose + 25 mM L-glucose as osmolar control, 2) 30 mM D-glucose, 3) 30 mM D-glucose + 100 mM ruboxistaurin for PKC-β (both PKC-β and PKC-βII inhibition), 4) 5 mM D-glucose + 100 mM PMA for PKC activation, and 5) 5 mM D-glucose + 1 mM Nω-nitro-L-arginine methyl ester (L-NAME) for eNOS inhibition. These slices were loaded with 10 μM DAF-2 DA in their respective solutions for 30 min in dark conditions at 25°C, followed by rinsing with the respective solutions three times and kept on ice in dark conditions until imaged in 2 h. During confocal imaging, each slice was put into a chamber with at least 10 ml of media and warmed to 37°C by a heated water jacket on the microscope stage. Samples were excited with a 488-nm argon laser and the emission was detected at 510–550 nm for DAF-2 fluorescence with simultaneous transmitted light imaging by a separate detector. The confocal settings (laser power, pinhole size, detector gain) were set with a normal control sample to avoid saturation of the fluorescent signal and kept identical in the same experiment to get comparable results from samples exposed to different treatments. Calcein/AM (2 μM, non-ion-specific dye, Molecular Probes) was used as a dye-loading control for DAF-2 DA following the same experimental procedure.

**Image analysis and statistical methods.** Confocal images were analyzed with the Metamorph imaging analysis software (Universal Imaging, Downingtown, PA). Fluorescence intensity of a glomerulus was obtained after subtraction of the background intensity. Because the background intensity from a glomerulus before dye loading is very slow motion of fluid flowing through the test chamber because stopping the flow momentarily had no effect on the recorded electrical current.

**RESULTS**

**NO-sensitive microelectrode measurements of glomerular NO production.** Figure 1 shows representative results of the microelectrode experiments during suppression of NO formation both with L-NAME and 30 mM D-glucose. Note that the initial NO recording was higher than the longer term recording after slightly withdrawing the microelectrode. This and many similar observations demonstrated that location in glomerulus influenced the NO measured. Consequently, in studies of this type, the electrode once placed should be kept at a constant location unless a particularly useful anatomic landmark is available for reference. In general, the use of hydraulic manipulators with no mechanical drift of their position and a vibration-free table was required to obtain highly stable recordings. The addition of 0.5 mM L-NAME (NOS inhibitor) inhibited the NO production from the glomerulus in as short as 10–15 min, but 30 min of exposure was more reliable. Exposure of glo-

![Fig. 2](http://ajprenal.physiology.org/)
meruli to 30 mM D-glucose, as shown in Fig. 1B, caused as rapid and as severe depression of the [NO] as l-NAME. Although there is a report that high concentrations of D-glucose can directly scavenge NO (8), we found the [NO] remained decreased after the D-glucose was removed by washout from the bath.

As microelectrodes were highly sensitive to changes in bath temperature, the slow changes in [NO] during perturbations in Fig. 1 reflected the gradual replacement of the bathing fluid contained pharmacological agents rather than the time-dependent ability of the tissue or microelectrode to respond to a perturbation. We were also concerned that something in the tissue other than NO could activate the electrodes. As has been reported by our previous study using other tissues (6), we rapidly froze and rewarmed the tissue slices to kill and freeze-fracture cell membranes. After being rewarmed, the electrodes did not detect any signal other than the brief transients associated with the microelectrode tip touching the tissue surface. Therefore, any chemicals present in cells before and after freezing that might leak into the interstitium were unlikely to contribute to the signal recorded during in vitro conditions. The NO-sensitive microelectrodes were unable to respond to D-glucose in our calibration cells, and we assumed this lack of response transferred to the in vitro environment.

In Fig. 2, the [NO] was measured at rest and during topical exposure to 1 µM bradykinin, 0.5 mM l-NAME, and 0.5 mM l-NAME + 1 µM bradykinin. At rest, we found the vast majority of glomerular [NO] was between 200 and 250 nM, but only if 100 µM l-arginine, the typical rodent plasma concentration, was present. In the absence of l-arginine, [NO] is much lower and decreased quite rapidly after the tissues were warmed to 37°C. We did detect low concentrations (<100 nM) of NO from microvessels and some of the renal tubules in the kidney slices, as might be expected because immunocytochemistry revealed eNOS in endothelia in or near these structures. However, the glomerular tissues generated such high [NO] by comparison that we concentrated on their NO-regulatory system. Bradykinin (1 µM) topically applied to the kidney slices routinely caused about a 50% increase in [NO] that was sustained so long as bradykinin was present. l-NAME was used to suppress any NOS in the preparation. The averaged results of seven animals indicated that 30 min of l-NAME treatment caused the glomerular NO production to decrease ~50% (Fig. 2). After exposure to l-NAME, bradykinin was unable to stimulate an increase in the glomerular [NO].

In eight kidney slices from four separate animals, the glomerular [NO] was measured at rest with 5 mM D-glucose and 30 min after exposure to 30 mM D-glucose. The microelectrode was placed on a glomerulus and not moved thereafter during the protocol. In every case, there was a progressive decline in the [NO] once D-glucose solution was applied over the tissues (Fig. 3) over the following 15–30 min of exposure. The average decline in [NO] with D-glucose was actually greater than with l-NAME.

**NOS expression in normal glomeruli.** These studies using the three NOS antibodies were performed on sections of kidneys from three mice with comparable results in each animal. To identify the sources of NO from NOS expression in normal glomeruli, we performed immunofluorescent staining on normal mouse kidney with specific antibodies to eNOS, nNOS, and iNOS, respectively. Glomeruli were always identified by the eNOS-specific antibody with positive staining in the endothelial cells of the glomerular capillaries (Fig. 4A). When treated with nNOS-specific antibody, the positive staining was only seen in the macula densa in the vascular pole of glomeruli but not inside glomeruli. In comparison, iNOS-specific antibody did not show positive staining either in the glomeruli or in renal tubule epithelial cells.

**DAF-2 and confocal imaging show that glomeruli have active NO production.** To confirm the microelectrode measurements and further differentiate spatial distribution of NO production by glomerular eNOS and possibly of significant NO production by nNOS of macula densa, we used confocal microscopy imaging on the renal slices loaded with DAF-2 DA. The imaging analysis allowed high spatial resolution as to specific cellular sites of NO formation. In addition, the optical method allowed multiple glomeruli to be studied in the same experiment.

Confocal imaging showed that DAF-2 DA-loaded samples had bright fluorescence from glomeruli and vascular endothelial cells (Fig. 5A) compared with an absence or very low fluorescence from tubular and parenchymal tissues. Figure 5B demonstrates the fluorescence due to calcein/AM (nonion sensitive) in all cells compared with the brighter fluorescence of DAF-2 DA activated by NO in glomerular cells. Figure 6A is a confocal image that demonstrated glomeruli were bright due to NO formation, whereas renal tubules were not at normal conditions. We confirmed that NO generation in cells loaded with the DAF-2 dye could be blocked by pharmacological suppression of eNOS with 1 mM l-NAME. As shown in Fig. 6B, very low fluorescence due to NO formation was found in l-NAME-treated tissue. Then this same tissue slice was exposed to 1 mM sodium nitroprusside to confirm all cells were loaded with DAF-2 and the dye would detect NO from decomposition of nitroprusside. As shown in Fig. 6C, both the glomerular and tubular cells had bright DAF-2 fluorescence in the presence of exogenous NO. These results indicated that DAF-2 can report cellular NO from either endogenous or...
exogenous NO sources. The limitation of a DAF-2 signal to glomeruli in Figs. 5A and 6A indicated little diffusion of NO from its site of production. As the DAF molecule is reported to respond to NO concentrations as low as 5 nM, diffusion of small concentrations of NO can be evaluated because all cells in the kidney take up the DAF dye as shown in Fig. 6C.

High glucose inhibits glomerular NO production, and PKC-β activity mediates the inhibition. We studied the effect of hyperglycemic d-glucose on glomerular NO production with the imaging method. The microelectrode studies in Figs. 1 and 3 indicated that high glucose suppressed NO formation, and we wished to confirm this observation with an alternative system, the DAF-2 reaction to NO. In addition, ruboxistaurin (previously LY-333531) was available to suppress PKC-β (both PKC-βI and PKC-βII). This drug has been shown to improve peripheral vascular function during diabetes mellitus (29), including some renal tissues (23) in chronic studies, and acutely can protect in vivo endothelial cells from hyperglycemia (7, 36). This allowed us to determine if PKC-β might be responsible for suppressed NO production when glomerular

Fig. 4. Immunofluorescence of NOS isoform expression in normal renal cortex. Cryosections of normal mouse kidney were treated by specific antibodies to eNOS (A), neuronal (n)NOS (B), inducible (i)NOS (C), and pre-immune serum (D), respectively. The red fluorescence is propidium iodide staining of the cell nucleus. Fluorescence (Alexa 488)-labeled secondary antibody binding to primary antibodies was used to visualize NOS localization (green). Based on 3 mice and multiple kidney slices from each mouse, the consistent finding was eNOS as the only NOS expressed in normal glomeruli (A), nNOS was present in only the macula densa (arrow in B), and iNOS did not express in the normal glomeruli (C). Bar = 20 μm. g, glomeruli.

Fig. 5. Glomeruli were the major structures showing strong NO production visualized by confocal imaging of 4,5-diaminofluorescein diacetate (DAF-2 DA)-loaded kidney slice. A: glomeruli and endothelial cells of arterioles (arrow) were positive for NO. Epithelial cells of renal tubules were generally negative in DAF-2 DA-loaded slices. B: calcine/AM-loaded sample from the same mouse showed homogeneous intracellular green fluorescence in all cells of the slice. Bar = 25 μm.
endothelial cells are exposed to high glucose. Examples of the images collected are shown in Fig. 7, and the analysis of these images is presented in Fig. 8 in terms of relative intensity measurements of DAF-2 fluorescence. We first tested that L-glucose would not cause a suppression of the NO signal, as shown in Fig. 7A. Even 25 mM L-glucose with 5 mM D-glucose for metabolic support did not impair generation of NO monitored by DAF-2 fluorescence. However, as shown in Fig. 7B, 30 mM D-glucose severely suppressed NO formation as registered by DAF-2. On a quantitative basis, the DAF-2 fluorescence intensity during 30 mM D-glucose was ~30% of that under normal conditions, as shown in Fig. 8. This relative reduction in NO formation during high glucose with dye is comparable to that recorded with microelectrodes shown in Fig. 3. Figure 7C presents an image of DAF-2 fluorescence during 30 mM D-glucose following treatment with 100 nM ruboxistaurin. The protected glomerular cells made NO despite the presence of high glucose. The third bar from the left in Fig. 8 presents the relative NO during the 30 mM D-glucose following treatment with 100 nM ruboxistaurin. The relative intensity was equivalent to normal conditions because PKC suppression protected NO formation during exposure to high glucose.

Fig. 6. Confocal imaging (merged images of fluorescence and transmitted light) of DAF-2 DA-loaded renal slices at rest and with l-NAME blockade of eNOS function. A: typical fluorescence due to NO production. B: tissue slice was exposed to 1 mM l-NAME, which effectively inhibited glomerular NO production. C: same tissue slice inhibited by the l-NAME that became bright after exposed to exogenous NO from 1 mM sodium nitroprusside (SNP). This indicated that l-NAME-treated tissues had loaded DAF-2 DA but the dye was not activated because it lacked biologically generated NO. This experiment was reproduced with similar results in tissue slices from 5 mice. Bar = 20 μm. D-glu, D-glucose.

Fig. 7. Images of events shown in quantitative form in Fig. 8 using DAF-2 DA dye to monitor NO production. A and B: glomeruli exposed to 5 and 30 mM D-glucose, respectively, to demonstrate [NO] change under these conditions as monitored with DAF-2 dye. C: tissue was treated with 100 nM ruboxistaurin before exposure to 30 mM D-glucose and NO formation was present. D: tissue was exposed to 100 nM PMA to activate PKC and NO production was strongly suppressed. E: when tissues exposed to PMA were then exposed to exogenous NO from nitroprusside, the fluorescence of DAF-2 was found. This indicated that PMA did not interfere with DAF-2 loading and sensing intracellular [NO]. Bar = 20 μm.
Inhibition of glomerular eNOS and NO production by PKC at high glucose. With two independent methods, the microelectrode measurements and NO-sensitive fluorescent dye with confocal microscopy, we showed that glomeruli have active in vitro NO production at a physiological d-glucose of 5 mM when sufficient l-arginine is present. As shown in Figs. 2, 6, and 8, eNOS inhibition with l-NAME caused a 50–60% decrease in the NO signal measured both by microelectrodes and DAF-2. In addition, before l-NAME, bradykinin could cause a large increase in [NO] that was fully blocked by l-NAME (Fig. 2). NO formation was also very vulnerable to acute 30 mM d-glucose and the NO signal as measured by either microelectrode or DAF-2 was reduced 60–70% (Figs. 3, 7, and 8).

The third hypothesis of the introduction that the suppression of NO by high glucose was associated with PKC activity in the glomerular endothelial cells was verified by two lines of evidence. First, PMA was used to activate PKC and the NO signal monitored with DAF-2 was decreased (Figs. 7 and 8). Second, ruboxistaurin was used to suppress PKC-β activity that is associated with hyperglycemia (23, 29) and this prevented the decline in NO signal measured with DAF-2 (Figs. 7 and 8). These results are quite similar to what has been found by our previous in vivo studies of arteriolar production of NO during hyperglycemia (7). In those studies in the rat in vivo microvasculature with 15–25 mM d-glucose decreased the [NO] by about one-half in 30–45 min and vessels had a depressed dilation to topical acetylcholine or bradykinin. Ruboxistaurin given before hyperglycemia protected the NO formation at rest and during receptor activation with acetyl-
choline and bradykinin in the in vivo studies. This pattern of response in the in vivo arterioles and that of the in vitro glomerular NO formation during high glucose and PKC blockade (Fig. 8) is quite similar. Therefore, the endothelial NO function in slices of renal tissue appeared to follow a pattern in which hyperglycemia rapidly suppressed eNOS function through a PKC mechanism quite like that in most other vascular beds. The results of our current study are also consistent with other reports indicating that transient hyperglycemia caused an increase of PKC activity in mouse embryos (21a), human platelets (1a), and mesangial cell cultures (1b).

Mechanisms related to PKC-dependent eNOS inhibition have been explored by recent studies. Several reports indicated that PKC mediates phosphorylation of Thr495 (bovine, human, porcine, and rabbit endothelial cells) (16, 33, 34, 37) or Thr497 (bovine endothelial cells) (35) in eNOS calmodulin-binding domain correlated to inhibition of eNOS function and decreased NO production. In addition, PKC may inhibit l-arginine transport into endothelial cells causing decreased NO production in endothelial cells (49). Although a complete picture about mechanisms of PKC activation on eNOS function and NO production is unclear, it is evident from our NO measurements with DAF-2 dye that PKC activation with PMA will cause suppression of eNOS function. Our results further show for the first time that high glucose inhibits glomerular eNOS-mediated NO production through a PKC mechanism in a renal slice of a normal animal.

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