High Glucose Augments Arginase Activity and Nitric Oxide Production in the Renal Cortex

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To clarify the interaction between arginase and nitric oxide (NO) production in the kidney with normal and high glucose levels, renal cortical slices from male Sprague-Dawley rats were incubated in Hank's solution containing various concentrations of L-norvaline (Nval; an arginase inhibitor), 500 U/mL superoxide dismutase, and either 5 mmol/L (normal) or 20 mmol/L (high) glucose (n = 5 per group). Incubation with Nval increased renal cortical NO_X (nitrite + nitrate) production dose-dependently, indicating competition between arginase and NO synthase (NOS) for the substrate (L-arginine). In the basal condition without Nval, high glucose also increased NO_X production to a rate 3 times that observed during incubation with normal glucose ($P < .01$). This effect of high glucose was not altered by Nval. Rather, the effects of high glucose and Nval were additive, indicating that the activity of NOS per se is enhanced by high glucose. Direct assay of arginase and NOS activities confirmed stimulation of both enzymes under the high glucose condition ($P < .05$, $P < .01$, *v* normal glucose, respectively). However, high glucose did not change the amount of L-arginine present in renal cortical slices. These data reveal that arginase competes with NOS for L-arginine in the renal cortex, and that high glucose increases the activity of both enzymes without affecting the amount of substrate. These results suggest that increased NOS activity, rather than altered substrate availability, may be the principal factor underlying increased NO synthesis in diabetic kidneys.

NITRIC OXIDE (NO) is well known as the endothelium-derived relaxing factor (EDRF) that has multiple physiological and pathophysiological effects.[1] In the kidney, NO has been shown to be involved in the control of arteriolar tone, nephron plasma flow, glomerular filtration rate, mesangial cell proliferation, tubular sodium handling, and maintenance of sodium balance.[2-4] Thus, regulation of NO production is of paramount importance for the kidney to maintain its function, especially in light of the fact that NO elicits detrimental effects under some pathophysiological conditions. For example, peroxynitrite ($ONOO^-$), produced by reaction between NO and superoxide anion (O_2^-), induces cellular and tissue injury by way of protein tyrosine nitration.[5-7] It has also been suggested that regulation of NO synthesis is deranged in diabetes mellitus (DM)[8] and that NO may provoke glomerular hyperfiltration in the earliest stages of DM,[9] which could represent a major factor contributing to the development of diabetic nephropathy. Indeed, we previously reported that renal cortical NO synthase (NOS) activity is increased under the high glucose condition associated with DM, although no change in protein level was evident for any of the 3 NOS isoforms.[10] Thus, the mechanism underlying altered NOS activity in the high glucose environment remains uncertain.

Numerous factors can influence NOS activity under physiological and pathophysiological conditions. A change in protein expression represents the most widely explored process, although NOS activity also is subject to post-translational regulation. Moreover, cofactor and/or substrate (L-arginine) availability can influence NO synthesis and may represent critical determinants of whether the enzymatic product of NOS activity is NO (adequate substrate) or O_2^- (limited substrate availability resulting in NOS uncoupling).[11] Arginine availability can be influenced by arginine transporters, citrulline-arginine recycling enzymes and hydrolysis to urea and ornithine by arginase.[12,13] Indeed, because NOS and arginase can compete for their common substrate, L-arginine,[14-17] an interaction between these 2 enzymes represents a potentially important factor in the regulation of NO production. One could envision a scenario in which suppression of arginase activity might augment L-arginine availability for NOS, thereby increasing NO synthesis; conversely, increased arginase activity could limit NO synthesis by reducing L-arginine availability for NOS. For example, arginase activity is 5-fold greater than NOS activity in isolated glomeruli,[18] and changes in the relative activities of these enzymes have been suggested to alter NO production in experimental glomerulonephritis.[19] Thus, it is important to understand the interaction between the arginase and NOS in the kidney and, in the context of the renal complications of DM, to determine if this interaction is dependent on glucose concentration.

To clarify these issues, studies were performed to examine the effect of an arginase inhibitor, L-norvaline (Nval), on NO production by renal cortical slices from rats under both normal and high glucose conditions. These studies were extended to include direct assay of NOS and arginase activities and tissue L-arginine concentration at different glucose levels.

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MATERIALS AND METHODS

Animals

The Kitasato University Institutional Animal Care and Use Committee approved all procedures used in this study. Male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing 250 to 350 g were assigned randomly to 1 of the 2 experiments described below (n = 5, each experiment). In each group, rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). The abdominal aorta was cannulated, allowing the kidneys to be flushed with heparinized saline. Each kidney was excised and weighed. Two medial slices were obtained from each kidney with a Stadie-Riggs microtome. The medullary portion of each slice was carefully removed and discarded. The cortical slices thus obtained were dissected and used for measurement of NO production, L-arginine concentration, arginase activity, and NOS activity.

Experiment 1

Renal cortical slices were incubated at 37°C for 90 minutes in Hank's balanced salt solution (HBSS) containing various concentrations of Nval (Sigma Chemical, St Louis, MO), 500 U/mL superoxide dismutase (SOD, O_2- scavenger; Sigma), and either 5 or 20 mmol/L glucose. The supernatant was removed at 90 minutes, and stored at −80°C until measurement of NO production. The renal cortex was weighed, minced, and homogenized (Ultra-Turrax T8; IKA Works, Staufen, Germany) in ice-cold HBSS. After 20 minutes treatment of the homogenate in 10 mmol/L 3-[(3-cholamidopropyl) dimethlammonio] propanesulfonic acid (CHAPS) at 4°C and subsequent centrifugation at 10,000 × *g*, samples were stored at −80°C until measurement of L-arginine and protein concentrations.

Total NO_X Production Assay

After the storage, supernatant was centrifuged at 10,000 × *g* at 4°C. NO_X (nitrite + nitrate) concentration in the centrifuged renal cortical supernatant was measured using the Griess assay (nitrite + nitrate assay kit;Dojindo Laboratories, Kumamoto, Japan). NO_X production was expressed as nanomoles per milligram renal cortical protein per 90 minutes.[20]

L-Arginine Assay

L-Arginine concentration was measured using the amino acid analyzer by high-performance liquid chromatography (HPLC) (Hitachi L-5800, Hitachi Ltd, Tokyo, Japan) with the o-phthalaldehyde method.[21] Briefly, stored supernatant of homogenized renal cortex was reacted in 1.5% sulfosalicylic acid and centrifuged at 10,000 × *g* at 4°C for 15 minutes. Supernatants were injected onto the amino acid analyzer. L-Arginine isolated by ion exchange column was reacted with o-phthalaldehyde and 2-mercaptoethanol, and measured based on fluorescence emission with an excitation wavelength of 360 nm and an emission wavelength of 470 nm.

Experiment 2

Renal cortical slices were incubated at 37°C for 90 minutes in HBSS containing 500 U/mL SOD and either 5 or 20 mmol/L glucose. After incubation, the renal cortical slices were used to determine NOS activity and arginase activity, and for protein assay.

NOS Assay

Total NOS activity in renal cortex was determined as the rate of L-[^{3}H]citrulline formation from L-[^{3}H]arginine, as described previously.[10,22] Briefly, the renal cortex was weighed, minced, and homogenized in ice-cold buffer containing 10 mmol/L HEPES, 320 mmol/L sucrose, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, 0.1 mmol/L soybean trypsin inhibitor, 0.1 mmol/L aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4). After 20 minutes treatment of the homogenate with 10 mmol/L CHAPS at 4°C and subsequent centrifugation at 10,000 × *g*, DOWEX (50WX8-400; Sigma) was employed to remove endogenous L-arginine from the supernatant. The supernatant was incubated in buffer containing 20 mmol/L HEPES, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 15 mmol/L NVal, 2 μmol/L BH_4, 1 mmol/L β-NADPH, 0.5 μmol/L calmodulin, 20 μmol/L L-arginine, and 0.2 μmol/L L-[2,3,4,5-^{3}H] arginine (0.82 μCi/mL assay buffer, 41 Ci/mmol specific activity; Amersham Pharmacis Biotech, Buckinghamshire, UK) (pH 7.2). Parallel reactions were performed in the presence of 300 μmol/L Nω-nitro-L-arginine (L-NNA; Sigma) to inhibit NOS activity. Samples were incubated 30 minutes at 37°C, after which the reaction was terminated by DOWEX treatment to remove L-arginine. L-[^{3}H]citrulline formed during the reaction was measured by liquid scintillation counting (LS 5000 Liquid Scintillation Systems; Beckman Instruments, Fullerton, CA). Total NOS activity of each background-corrected sample was determined as the difference between the radioactivity with and without 300 μmol/L L-NNA, and was expressed as picomoles L-[^{3}H]citrulline formed per hour per milligram protein.

Arginase Assay

Arginase activity in renal cortex was determined as the rate of urea formation from L-arginine, as described previously.[23] Briefly, the renal cortex was weighed, minced, and homogenized in ice-cold buffer containing 50 mmol/L Tris, 0.15 mmol/L pepstatin A, 0.15 mmol/L antipain, and 15 μmol/L aprotinin (pH 7.5). After 20 minutes treatment of the homogenate in 10 mmol/L CHAPS at 4°C and subsequent centrifugation at 10,000 × *g* at 4°C, samples of supernatant were stored at −80°C until arginase and protein assay.

For arginase activity assay, the supernatant was incubated in activation solution containing 5 mmol/L $MnCl_2$, 50 mmol/L Tris (pH 7.5) for 10 minutes at 55°C. The arginase reaction (arginine hydrolysis) was performed in 50 mmol/L Tris and 250 mmol/L arginine (pH 9.7) for 60 minutes at 37°C. The reaction was stopped by acid mixture containing H_2SO_4, H_3PO_4, and H_2O (1:3:7) and urea was quantified colorimetrically in 0.43% α-isonitrosopropiophenone at 100°C for 45 minutes, with absorbance measured at 540 nm in a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA) after 10 minutes in the dark. Arginase activity was reported as Nval-sensitive (15 mmol/L) urea production, corrected for results obtained in the absence of exogenous L-arginine (effect of endogenous L-arginine on arginase activity) and during NOS inhibition (10 mmol/L L-NNA).

Protein Assay

Protein concentration in homogenized renal cortex was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories).

Statistics

Data were analyzed by analysis of variance (ANOVA) for repeated measures or unpaired *t* tests, as appropriate. *P* values less than .05 were considered significant. All data are reported as means ± SE.

RESULTS

NO_X Production by Renal Cortical Slices in Normal (5 mmol/L) and High (20 mmol/L) Glucose

Incubation of renal cortical tissue in media containing 20 mmol/L glucose caused a 160% increase in NO_X production, relative to that evident during incubation in 5 mmol/L glucose ($P < .01$; Fig 1A).

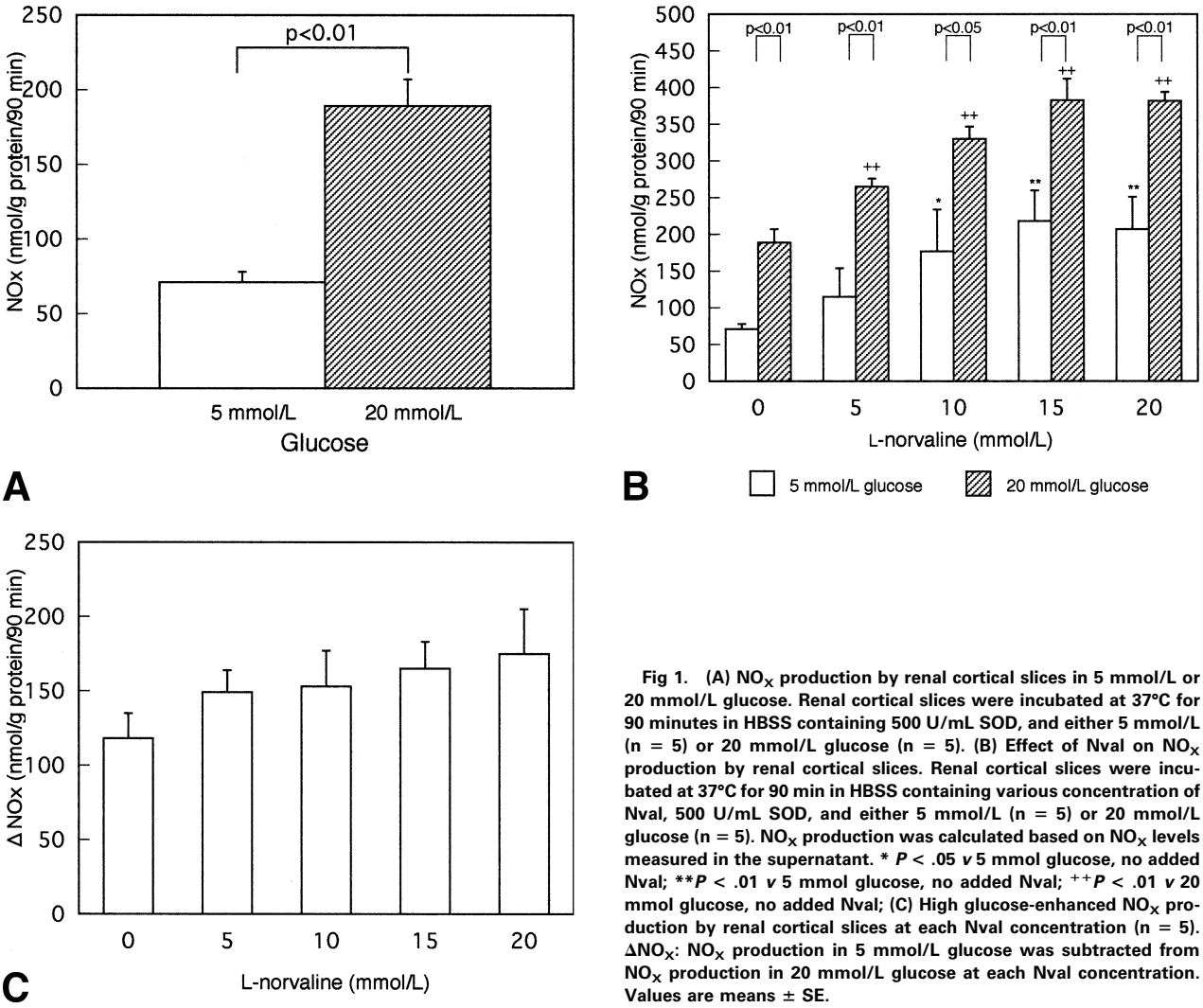

Fig 1. (A) NO_X production by renal cortical slices in 5 mmol/L or 20 mmol/L glucose. Renal cortical slices were incubated at 37°C for 90 minutes in HBSS containing 500 U/mL SOD, and either 5 mmol/L (n = 5) or 20 mmol/L glucose (n = 5). (B) Effect of Nval on NO_X production by renal cortical slices. Renal cortical slices were incubated at 37°C for 90 min in HBSS containing various concentration of Nval, 500 U/mL SOD, and either 5 mmol/L (n = 5) or 20 mmol/L glucose (n = 5). NO_X production was calculated based on NO_X levels measured in the supernatant. * $P < .05$ *v* 5 mmol glucose, no added Nval; ** $P < .01$ *v* 5 mmol glucose, no added Nval; $^{++}P < .01$ *v* 20 mmol glucose, no added Nval; (C) High glucose-enhanced NO_X production by renal cortical slices at each Nval concentration (n = 5). ΔNO_X: NO_X production in 5 mmol/L glucose was subtracted from NO_X production in 20 mmol/L glucose at each Nval concentration. Values are means ± SE.

Effect of L-Norvaline on NO_X Production by Renal Cortical Slices

Figure 1B illustrates the effects of 0 to 20 mmol/L Nval on NO_X production by renal cortical slices incubated with either 5 mmol/L glucose (n = 5) or 20 mmol/L glucose (n = 5). Addition of Nval increased NO_X production by renal cortical slices significantly and dose-dependently, up to 15 mmol/L of Nval, and this effect was evident in both 5 and 20 mmol/L glucose media. As arginase and NOS are known to share a common substrate, L-arginine, these results suggest that inhibition of arginase by Nval increases NO synthesis via increased availability of the substrate for NOS. It was also suggested that in this experimental setting, maximum inhibition of arginase was obtained with 15 mmol/L Nval.

In an effort to further assess the interaction between arginase inhibition and high glucose on enhanced NO_X production by renal cortical slices, the amount of NO_X production with 5 mmol/L glucose was subtracted from that with 20 mmol/L glucose at each Nval concentration (ΔNO_X, Fig 1C). Regardless of the degree of arginase inhibition by Nval, the glucose-induced enhancement of NO_X production was almost constant (~150 nmol/g protein/90 min). These data indicate that the effect of high glucose on NO synthesis is additive to, not synergistic with, the effect of arginase inhibition.

Effect of Glucose on Renal Cortical NOS Activity, Arginase Activity, and L-Arginine Concentration

Figure 2A summarizes renal cortical NOS and arginase activities under 5 mmol/L (n = 5) and 20 mmol/L (n = 5) glucose conditions. From this figure, it is evident that the activities of both enzymes were significantly stimulated by 20 mmol/L glucose (NOS, $P < .01$; arginase, $P < .05$). However, this activation of the 2 enzymes could not be attributed to the increment of their substrate, because the amount of renal cortical L-arginine available in the tissue did not differ in 5 or 20 mmol/L glucose conditions (Fig 2B).

DISCUSSION

Clarification of the development and pathophysiology of diabetic nephropathy is one of the major objectives in current

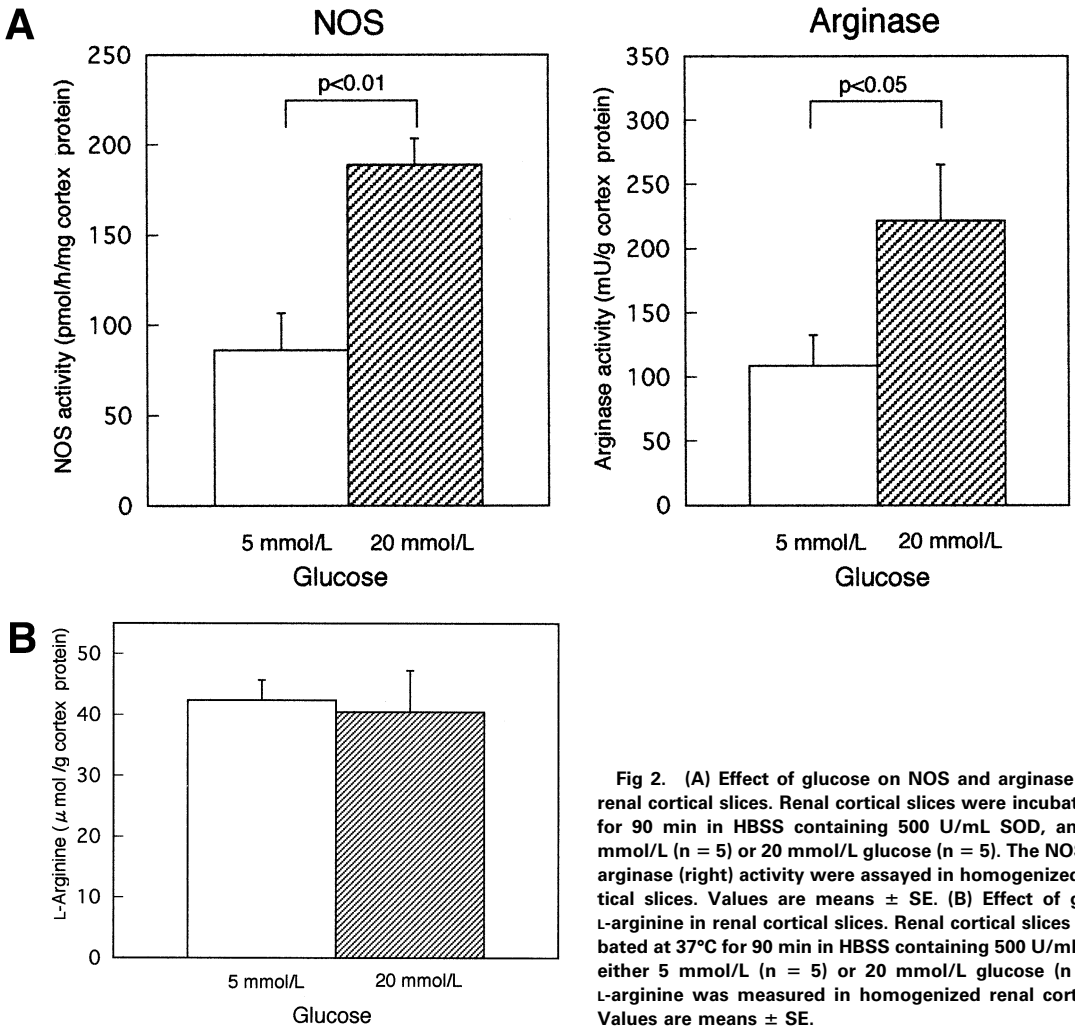

Fig 2. (A) Effect of glucose on NOS and arginase activity in renal cortical slices. Renal cortical slices were incubated at 37°C for 90 min in HBSS containing 500 U/mL SOD, and either 5 mmol/L (n = 5) or 20 mmol/L glucose (n = 5). The NOS (left) and arginase (right) activity were assayed in homogenized renal cortical slices. Values are means ± SE. (B) Effect of glucose on L-arginine in renal cortical slices. Renal cortical slices were incubated at 37°C for 90 min in HBSS containing 500 U/mL SOD, and either 5 mmol/L (n = 5) or 20 mmol/L glucose (n = 5). The L-arginine was measured in homogenized renal cortical slices. Values are means ± SE.

nephrology. Recently, much attention has been focused on NO, as the diversity of its intrarenal action has been recognized and a derangement of NO synthesis regulation in DM has become evident. We previously reported that both NOS and SOD activities were accelerated in the renal cortex of rats with DM.[10] We have also reported that protein tyrosine nitration, likely via ONOO− formation, accompanies increased NO and O2− production in the rat cortex during the early stage of DM, which may lead to renal damage.[10] Komers et al[24] have shown that NOS inhibition normalized the glomerular filtration rate in DM rats, suggesting that the glomerular hyperfiltration characteristic of the early stage of DM may be engendered through arteriolar dysfunction due to a deranged NO system. However, renal microvessels from DM rats exhibit diminished NO-dependent basal tone and loss of the modulatory influence of endogenous NO on agonist-induced constriction.[25,26] In light of these observations, and because NO appears to have both beneficial and detrimental effects on renal function, it is of paramount importance to understand how the increased extracellular glucose levels that accompany DM might influence NO production and potentially contribute to the development of diabetic nephropathy.

Several recent reports have indicated a possible interaction between arginase and NOS.[14-17] In mammalian cells, L-arginine is synthesized primarily by argininosuccinate synthase and argininosuccinate lyase (forming the citrulline-NO cycle), and catabolized by 2 arginase isoforms (type I, II) and 3 NOS isoforms (neuronal NOS, nNOS,or NOS1; inducible NOS, iNOS or NOS2; and endothelial NOS, eNOS or NOS3). Among these enzymes, type II arginase and NOS play predominant roles in renal L-arginine catabolism.[27-31] Therefore, arginase may compete with NOS for intracellular L-arginine, and tissue NO production may be affected through an interaction between the 2 enzymes. To clarify this issue and its potential impact on the kidney in DM, the present study was designed to examine the effect of an arginase inhibitor (Nval) on NO production by rat renal cortical slices under both normal and high glucose conditions.

Using Nval as an inhibitor of arginase, we confirmed that there is competition for the common substrate (L-arginine) between arginase and NOS in the renal tissue. We also demonstrated that the activities of both enzymes were increased under high glucose conditions. Despite activation of competing enzyme, the production of NO was increased significantly

under high glucose conditions. This situation likely reflects different affinities of the 2 enzymes for the common substrate. While the Km of various NOS isoenzymes for L-arginine is in the 2 to 20 μmol/L range, the Km of mammalian arginase is around 2 to 20 mmol/L.[17,32-34] Thus, the affinity of NOS for L-arginine is approximately 1,000-fold greater than that of arginase. Moreover, the renal cortical L-arginine levels did not differ significantly between normal and high glucose conditions, suggesting the relative abundance of the substrate for these 2 enzymes. Thus, even though both enzymes were activated by high glucose conditions, total substrate availability appeared sufficient to sustain these activation levels. However, the technical limitation that the L-arginine concentration of the homogenated renal cortex may not precisely equivalent to the intracellular availability of the substrate should also be considered.

In previous studies, we used Western blot analysis to determine if a change in protein levels of one or more NOS isoforms might be responsible for the increase in overall NOS activity evident in the kidneys of rats with streptozotocin (STZ)-induced DM.[10] In that study, densitometric analysis failed to unveil a statistically significant increase in any NOS isoform in kidneys from STZ rats, indicating that the enhanced NOS activity in the hyperglycemic condition of DM (2 weeks after onset) could not be attributed to increased expression of the enzyme. In the present study, NOS activity in the soluble fraction of renal cortical slices was measured after 90 minutes exposure to glucose concentrations similar to those found in DM. Our observations reveal that a high glucose environment is sufficient to increase renal cortical NO production and NOS activity, suggesting that this process may be responsible for the increased NOS activity in DM. Because eNOS is the predominant NOS isoform in the soluble fraction of renal cortex,[10] this isoform likely is responsible for the NOS activation events observed in the present study.

The mechanism underlying activation of renal cortical NOS activity under high glucose conditions is uncertain, although the 90-minute time frame makes it unlikely that protein synthesis is involved. An osmotic effect of 20 mmol/L glucose is also unlikely, as previous reports have documented the inability of 50 mmol/L mannitol to mimic the ability of high glucose to rapidly increase NO production by glomerular endothelial cells.[35] The activity of eNOS is influenced by multiple processes, including post-translational modifications of the enzyme, protein-protein interactions, cofactors and prosthetic groups, calcium/calmodulin, and phosphorylation. Unlike other NOS isoforms, eNOS is localized within caveolae[36,37]—specialized invaginations of the plasma membrane that are prominent in endothelial cells and enriched with caveolin. Parat et al[38] recently reported that 20 minutes exposure to H_2O_2 inhibits palmitoylation of caveolin-1, a process critical to targeting of this protein to caveolar domains.[37] As eNOS activity is inhibited by interaction with caveolin-1 in raft domains,[39] and in light of our previous observation that 90 min utes exposure to high glucose media stimulates O_2- production by renal cortical slices,[40] impaired caveolin trafficking to the cell membrane might underlie the glucose-induced activation of NOS under our experimental conditions. Caveolae also contain G-protein–coupled cell surface receptors, receptor tyrosine kinases, Ca^{2+} pumps and channels, protein kinases (A, B, C, and D), and cationic amino acid transporters.[41-44] Regulation of eNOS activity by a complex balance of phosphorylation reactions at several serine and threonine residues[45-47] raises the possibility that protein kinase activation under high glucose conditions might contribute to increased NOS activity. Indeed, short-term (15 to 60 minutes) exposure of endothelial or proximal tubule cells to a high glucose environment increases cyclic adenosine monophosphate (cAMP) levels and triggers protein kinase A–and C–dependent processes.[48,49] Moreover, multiple protein kinase C isoforms are activated within 5 minutes of H_2O_2 exposure,[50] raising the possibility that glucose-induced oxidative stress could trigger a cascade of signaling events with the capacity to influence NOS activity over the 90-minute time frame of our experiments.

Enhancement of substrate transportation is another mechanism through which high glucose might accelerate NO production by eNOS. L-Arginine can be transported into mammalian cells by a number of different cationic amino acid transporter systems (y^+, $b^{0,+}$, $B^{0,+}$, and L).[51,52] L-Arginine transport into endothelial cells is mediated by both the Na^+-dependent active (system $B^{0,+}$) and the Na^+-independent (system y^+) transport processes. However, 80% of L-arginine uptake into endothelial cells occurs via system y^+, which is localized with eNOS in caveolae.[53,54] Therefore, endothelial cells mostly rely on system y^+ for their supply of L-arginine. Using human endothelial cells, Sobrevia et al[55,56] have recently shown that high glucose activates both system y^+ and NOS activity. In our study, the total amount of renal cortical L-arginine did not decrease despite of the activation of both enzymes (arginase and NOS), suggesting the abundance of the substrate in the current settings. This result favors the contention that enhancement of NOS activity, rather than altered substrate availability, is the principal factor promoting increased NO production under high glucose conditions. However, because regulation of substrate transport plays a major role in determining NOS activity in vivo, we cannot rule out the possibility that high glucose increases NO production through enhancement of substrate transport or distribution of arginine to a distinct intracellular pool in vivo,[54] where it might support activation of both NOS and arginase.

In summary, the present study revealed that NOS competes with arginase for L-arginine in the renal cortex. The study also confirmed that high glucose stimulates renal cortical NO production. However, the enhancing effect is additive, not synergistic, to that of arginase inhibition. Because the activity of NOS per se was enhanced, it was concluded that increased renal NO production in DM is not due to the altered balance between the 2 competing enzymes. Moreover, this study also revealed relative tissue abundance of L-arginine during the activation of the both enzymes. These observations make it unlikely that tissue NO levels are controlled indirectly through modulation of arginase or L-arginine availability in the renal cortex, although a redistribution of arginine cannot be ruled out. In order to prevent the detrimental effect of excessive NO production in DM, modulation of NOS activity per se or scavenging of excess NO in the tissue seems to be essential.

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