Role of renal cortical cyclooxygenase-2 expression in hyperfiltration in rats with high-protein intake

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Yao, Bing, Jie Xu, Zhonghua Qi, Raymond C. Harris, and Ming-Zhi Zhang. Role of renal cortical cyclooxygenase-2 expression in hyperfiltration in rats with high-protein intake. Am J Physiol Renal Physiol 291: F368–F374, 2006. First published May 16, 2006; doi:10.1152/ajprenal.00500.2005.—Renal cortical cyclooxygenase-2 (COX-2) is restricted to the macula densa and adjacent cortical thick ascending limbs (MD/cTALH). Renal cortical COX-2 increases in response to diabetes and renal ablation, both of which are characterized by hyperfiltration and reduced NaCl delivery to the MD due to increased proximal NaCl reabsorption. High-protein intake also induces hyperfiltration and decreases NaCl delivery to the MD due to increased NaCl reabsorption proximally. We investigated whether high protein induces cortical COX-2 and whether cortical COX-2 contributes to high protein-induced hyperfiltration and increased intrarenal renin biosynthesis. Cortical COX-2 increased after protein loading but decreased after protein restriction. COX-2 inhibition attenuated high protein-induced hyperfiltration but had no effect on high protein-induced intrarenal renin elevation. Therefore, induction of cortical COX-2 contributed to high protein-induced hyperfiltration but not intrarenal renin elevation. In the kidney cortex, neuronal nitric oxide synthase (nNOS) is also localized to the MD, and interactions between intrarenal nNOS and COX-2 systems have been proposed. Cortical COX-2 elevation seen in salt restriction was blocked by nNOS inhibitor. Cortical nNOS expression also increased after protein loading, and inhibition of nNOS activity completely reversed high protein-induced cortical COX-2 elevation and hyperfiltration. These results indicate that NO is a mediator of high protein-induced cortical COX-2 elevation and suggest that both intrarenal nNOS and COX-2 systems appear to regulate afferent arteriolar tone and subsequent hyperfiltration seen in high-protein intake.

previouS reports indicate that dietary protein excess accelerates, and dietary protein restriction retards, the progression of chronic renal failure (11, 15, 24, 29, 39). Both acute and chronic protein intake lead to increases in renal blood flow and glomerular filtration rate (GFR). In micropuncture studies in the rat, Meyer et al. (23) found that the increased GFR in response to amino acid infusion was associated with a reduction in afferent arteriolar resistance and a subsequent increase in single-nephron plasma flow. The physiological mechanisms responsible for these changes in renal hemodynamics after protein loading are still not well understood.

Prostaglandins regulate vascular tone and salt and water homeostasis in the mammalian kidney. Renal prostaglandin production increases in response to protein load or amino acid infusion and decreases in response to protein restriction (8, 14, 22, 23, 29, 33). Numerous studies have shown that inhibition of prostaglandin production by nonsteroidal anti-inflammatory drugs (such as aspirin and meclofenamate) abolishes the normal increase in GFR after a meat meal or during amino acid infusion in human subjects and animals (14, 22, 33, 36). Therefore, it has been suggested that the increased synthesis of vasodilatory prostaglandins such as PGE2 and PGI2 may contribute to alterations in renal hemodynamics in response to a protein load.

Cyclooxygenase (COX) is the rate-limiting enzyme for prostaglandin production. COX exists in two isoforms: constitutive COX-1 and inducible COX-2 (12). In mammalian kidney cortex, COX-2 is expressed in the macula densa and adjacent cortical thick ascending limb of Henle (MD/cTALH) (13, 40). In the mammalian kidney, the MD is involved in regulation of tubuloglomerular feedback (TGF) and renin release. Alteration of luminal NaCl delivery to the MD is the signal for MD regulation of TGF and renin release, with increased salt delivery stimulating TGF and decreased salt delivery stimulating renin release (2, 12, 32, 35). High-protein intake has been reported to cause a selective hypertrophy of the thick ascending limb and increased NaCl reabsorption in this segment and/or in the proximal tubule, leading to reduced NaCl delivery to the MD (1, 31, 32, 36, 37). COX-2 expression in the MD/cTALH increases in conditions associated with reduced NaCl delivery to the MD (18–20, 32, 34, 41). COX-2 expressed in the MD/cTALH has been proposed to attenuate TGF through dilation of afferent arterioles and to stimulate renin release (9, 12, 16). In the current studies, we tested the hypothesis that high-protein intake might lead to increased COX-2 expression in the MD/cTALH due to reduced NaCl delivery to the MD, and increased COX-2 expression might, in turn, contribute to the elevation of GFR.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (107–115 g at the beginning of the experiments) were used. The rats were treated with either a low-protein diet (CA170595, 8% casein, Harlan Teklad, Madison, WI), normal-protein diet (TD 91352, 20% casein, control), or high-protein diet (CA170598, 50% casein) for 2 wk. Subsets of rats on the high-protein diet were treated with either the selective COX-2 inhibitor SC-58236 (2 mg/kg, daily gastric gavage, gift from Searle Monsanto, St. Louis, MO) or the selective neuronal nitric oxide (NO) synthase (nNOS; inducible NOS I) inhibitor 7-nitroindazole (7-Ni; 20 mg/kg daily ip, Calbiochem, La Jolla, CA) during the second week of high-protein diet treatment.

Measurement of GFR. GFR was measured in conscious rats using FITC-inulin clearance as described by Qi et al. (26) with minor modifications. Briefly, dialyzed FITC-inulin (3.74 μl/g body wt) was injected into the femoral vein under light anesthesia induced by

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isoflurane (Baxter Pharmaceutical Products, Deerfield, IL). The anesthesia lasted 20 s. Approximately 50 μl of blood were collected via the femoral vein at 3, 7, 10, 15, 35, 55, and 75 min postinjection of FITC-inulin, yielding 20 μl of plasma for the determination of FITC concentration by fluorescence.

**Immunohistochemistry.** In general, at the termination of an experiment, one kidney from each rat was removed for Western blot analysis and the other was perfused with fixative in situ for histology. Under deep anesthesia with nembutal (70 mg/kg ip), the rats were first exsanguinated with 50 ml/100 g heparinized saline (0.9% NaCl, 2 U/ml heparin, 0.02% sodium nitrite) through a transcardial aortic cannula and fixed with 3.7% formaldehyde in an acidic solution (pH 4.5) containing phosphate, periodate, acetate, and sodium chloride as described (40). The fixed kidney was dehydrated through a graded series of ethanols, embedded in paraffin, sectioned (4 μm), and mounted on glass slides. Internal controls and comparisons were facilitated by creating compound blocks with multiple specimens that were sectioned and stained together. The kidney sections were immunostained with either affinity-purified rabbit polyclonal anti-murine COX-2 antibody (160126, Cayman Chemicals, Ann Arbor, MI) di- nostained with either affinity-purified rabbit polyclonal anti-murine COX-2 antibody (160126, Cayman Chemicals, Ann Arbor, MI) diluted to 2.5 μg/ml, or with affinity-purified rabbit polyclonal anti-nNOS antibody (61–7000, Zymed Laboratories, South San Francisco, CA) diluted to 1 μg/ml, or with polyclonal rabbit anti-renin antiserum (1–6,000 dilution, a generous gift from Prof. T. Inagami, Vanderbilt University). Vectastain ABC-Elite was used to localize the primary antibodies with a chromagen of oxidized diaminobenzidine, followed by a light toluidine blue counterstain.

**Immunoblotting.** Homogenates were prepared in 20 mM Tris·HCl, pH 8.0, with a protein inhibitor mixture (Boehringer Mannheim). After 10-min centrifugation at 10,000 g, the supernatant was centrifuged for 60 min at 100,000 g to sediment microsomes as described (13). A bicinchoninic acid (BCA) protein assay reagent kit was used to determine protein concentration. The microsomes were resuspended in homogenizing buffer, mixed with an equal volume of 2× SDS sample buffer, and boiled for 5 min. The proteins were separated on 10% SDS gels under reducing conditions and transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA). After being blocked with 20 mM Tris·HCl, pH 7.4/500 mM NaCl/5% nonfat milk/0.1% Tween 20 for 3 h at room temperature, the blots were incubated overnight at 4°C with affinity-purified rabbit polyclonal anti-nmurine COX-2 antibody (160126, Cayman Chemicals, Ann Arbor, MI) diluted to 2.5 μg/ml, or with affinity-purified rabbit polyclonal anti-nNOS antibody (61–7000, Zymed Laboratories, South San Francisco, CA) diluted to 1 μg/ml, or with polyclonal rabbit anti-renin antiserum (1–6,000 dilution, a generous gift from Prof. T. Inagami, Vanderbilt University). After being blocked with 20 mM Tris·HCl, pH 7.4/500 mM NaCl/5% nonfat milk/0.1% Tween 20 for 3 h at room temperature, the blots were incubated overnight at 4°C with affinity-purified rabbit polyclonal anti-nmurine COX-2 antibody (160126, Cayman Chemicals, Ann Arbor, MI) diluted to 2.5 μg/ml, or with affinity-purified rabbit polyclonal anti-nNOS antibody (61–7000, Zymed Laboratories, South San Francisco, CA) diluted to 1 μg/ml, or with polyclonal rabbit anti-renin antiserum (1–6,000 dilution, a generous gift from Prof. T. Inagami, Vanderbilt University). After being blocked with 20 mM Tris·HCl, pH 7.4/500 mM NaCl/5% nonfat milk/0.1% Tween 20 for 3 h at room temperature, the blots were incubated overnight at 4°C with affinity-purified rabbit polyclonal anti-nmurine COX-2 antibody (160126, Cayman Chemicals, Ann Arbor, MI) diluted to 2.5 μg/ml, or with affinity-purified rabbit polyclonal anti-nNOS antibody (61–7000, Zymed Laboratories, South San Francisco, CA) diluted to 1 μg/ml, or with polyclonal rabbit anti-renin antiserum (1–6,000 dilution, a generous gift from Prof. T. Inagami, Vanderbilt University). After being blocked with 20 mM Tris·HCl, pH 7.4/500 mM NaCl/5% nonfat milk/0.1% Tween 20 for 3 h at room temperature, the blots were incubated overnight at 4°C with affinity-purified rabbit polyclonal anti-nmurine COX-2 antibody (160126, Cayman Chemicals, Ann Arbor, MI) diluted to 2.5 μg/ml, or with affinity-purified rabbit polyclonal anti-nNOS antibody (61–7000, Zymed Laboratories, South San Francisco, CA) diluted to 1 μg/ml, or with polyclonal rabbit anti-renin antiserum (1–6,000 dilution, a generous gift from Prof. T. Inagami, Vanderbilt University). After being blocked with 20 mM Tris·HCl, pH 7.4/500 mM NaCl/5% nonfat milk/0.1% Tween 20 for 3 h at room temperature, the blots were incubated overnight at 4°C with affinity-purified rabbit polyclonal anti-nmurine COX-2 antibody (160126, Cayman Chemicals, Ann Arbor, MI) diluted to 2.5 μg/ml, or with affinity-purified rabbit polyclonal anti-nNOS antibody (61–7000, Zymed Laboratories, South San Francisco, CA) diluted to 1 μg/ml, or with polyclonal rabbit anti-renin antiserum (1–6,000 dilution, a generous gift from Prof. T. Inagami, Vanderbilt University).

**Quantitative analysis.** Western blots were quantified with an IS-1000 digital imaging system (Alpha Innotech, San Leandro, CA). The COX-2 immunoreactivity (-ir) band density from the control animal was designated as one, and that from the experimental animal was expressed as fold of control. On the basis of the distinctive density and color of nNOS-ir or renin-ir in video images, the number, size, and position of stained cells were quantified using the BICOQUANT true-color windows system (R & M Biometrics, Nashville, TN) equipped with a digital stage encoder that allows high-magnification images to be mapped to global coordinates throughout the whole section (40, 41). The whole renal cortex from each section was quantified at ×160 magnification. Sections from at least three regions of each kidney were analyzed, and the average was used as data from one animal sample.

**Microscopy.** Brightfield images from a Leitz Orthoplan microscope with a DVC digital RGB video camera were digitized by the BICOQUANT image-analysis system and saved as computer files. Contrast and color level adjustment (Adobe Photoshop) were performed for the entire image; i.e., no region- or object-specific editing or enhancements were performed.

**Statistical analysis.** All values are presented as means ± SE. ANOVA and Bonferroni t-test were used for statistical analysis, and differences were considered significant when P < 0.05.

**RESULTS**

**Protein intake and physiological parameters.** After treatment with different protein diets for 2 wk, the animals were killed, and the left kidney was removed, weighed, and frozen in liquid nitrogen for Western blot analysis; the right kidney was perfused with FPAS for COX-2, renin, and nNOS immunostaining. As expected, dietary protein content influenced animal growth and kidney growth (Fig. 1). The average body weight was significantly lower for rats on the low-protein diet than for rats on a normal-protein diet (“control”), whereas the average body weights of rats between normal-protein diet and high-protein diet groups were not different (control: 159.2 ± 21.5 g; low-protein diet: 123.2 ± 8.6 g, P < 0.01 vs. control; high-protein diet: 162.5 ± 17.6 g, NS vs. control; n = 6 in each group). The average left kidney weight was significantly lower for rats on the low-protein diet than for rats on a normal-protein diet (control: 0.61 ± 0.02 g; low-protein diet: 0.49 ± 0.02 g, P < 0.01 vs. control; n = 6 in each group). Although the high-protein diet did not significantly influence animal growth, it significantly augmented kidney growth (0.88 ± 0.03 g, P < 0.01 vs. control; n = 6). The average ratio of left kidney vs. body weight was significantly higher in rats on high-protein diet than rats on normal-protein diet (control: 0.00383 ± 0.00012; low-protein diet: 0.00398 ± 0.00016, NS vs. control; high-protein diet: 0.00542 ± 0.00018, P < 0.01 vs. control; n = 6 in each group), confirming that the high-protein intake caused kidney hyper trophy.
Protein intake and renal COX-2 expression. Renal cortical prostaglandin production is stimulated by high-protein intake but inhibited by protein restriction (8, 22, 29, 33). To investigate whether COX-2 and/or COX-1 expression in kidney cortex might contribute to variations in renal cortical prostaglandin production in response to alterations of protein intake, renal cortical COX-2 and COX-1 expression were evaluated by Western analysis and immunohistochemistry. As indicated in Fig. 2A, renal cortical COX-2 expression increased in response to high-protein intake but decreased in response to low-protein intake (fold of control: high-protein diet: 2.03 ± 0.11, *P* < 0.01 vs. control; low-protein diet: 0.31 ± 0.01, *P* < 0.01 vs. control; *n* = 6 in each group). Immunohistochemical localization indicated that detectable cortical COX-2 expression remained localized to the MD and surrounding cTALH cells and the number of COX-2-ir-positive cells in MD/cTALH increased in high protein-treated rats and decreased in low protein-treated rats (Fig. 3). In contrast, renal cortical COX-1 expression was not influenced by alterations in protein intake (Fig. 2).

High-protein intake has also been reported to increase urinary prostaglandin excretion (8, 33). The renal medulla is a major source of urinary prostaglandin production in normal adult rodents. To investigate whether alterations in renal medullary COX-2 and/or COX-1 expression might contribute to high protein-induced elevation of urinary prostaglandin excretion, renal medullary COX-2 and COX-1 expression was determined in rats with different protein intakes. As indicated in Fig. 2B, neither medullary COX-2 nor COX-1 expression was measurably different in rats on the different protein diets.

**COX-2 and GFR.** Either acute or chronic protein load increases GFR (1, 8, 21, 22, 29, 31, 33, 36, 37). To investigate
whether COX-2 expression in kidney cortex correlates with GFR variations in response to alterations in protein intake, GFR was measured in rats on the different protein diets (26). As expected, GFR was lowest in rats on the low-protein diet, intermediate in rats on the normal-protein diet, and highest in rats on the high-protein diet (control: 3.73 ± 0.32 ml/min/100 g/18528 kg; low-protein diet: 2.94 ± 0.31 ml/min/100 g/18528 kg; high-protein diet: 4.84 ± 0.79 ml/min/100 g/18528 kg, P < 0.05 vs. control; n = 5 in each group; Fig. 4). Therefore, alterations in COX-2 expression in the kidney cortex correlated with variations in GFR in response to alterations in protein intake. To investigate further this relationship, rats on the high-protein diet were treated either chronically (1 wk) or acutely (1 h) with the selective COX-2 inhibitor SC-58236 before measurement of GFR. Treatment with SC-58236 for a week did not influence animal growth or kidney growth in rats on the high-protein diet (body wt: 149.2 ± 21 g, NS vs. high-protein diet; left kidney weight: 0.85 ± 0.03 g, NS vs. high-protein diet; n = 6 in each group; Fig. 1). However, high protein-induced GFR elevation was completely reversed by either chronic or acute inhibition of COX-2 activity (high-protein diet plus SC-58236 for 1 wk: 3.23 ± 0.80 ml/min/100 g/18528 kg, P < 0.05 vs. high-protein diet but NS vs. control; high-protein diet plus SC-58236 for 1 h: 3.66 ± 0.41 ml/min/100 g/18528 kg, P < 0.05 vs. high-protein diet but NS vs. control; n = 5 in each group). Urine volume was not altered by chronic (1 wk) COX-2 inhibition (control: 10 ± 1 ml/24 h; high-protein: 13 ± 2 ml/24 h; high-protein + SC-58236: 13 ± 2 ml/24 h; n = 4).

Interactions between intrarenal cortical COX-2 and nNOS in rats with high-protein diet. It is well established that COX-2 and nNOS are colocalized to the MD (25, 35). Renal cortical COX-2 elevation in response to salt restriction was prevented by inhibition of nNOS activity (4). The effect of protein intake on renal cortical nNOS expression was determined by immunohistochemistry (Fig. 5) and quantified by image analysis. Renal cortical nNOS expression correlated with COX-2 expression in response to alterations of protein intake. Renal cortical nNOS expression was lowest in rats on the low-protein diet, intermediate in rats on the normal-protein diet, and high-

Fig. 5. Expression of nNOS (A-C) and renin (D-F) in kidney cortex. In control rats (A), nNOS-ir-positive cells were restricted to the MD (arrows). In rats on HP (B), increased nNOS-ir-positive cells were found in the MD (arrows) and adjacent cTALH (arrowheads). In rats on LP (C), decreased nNOS-ir-positive cells were detected in the MD (arrow). Renin-ir was higher in high-protein diet-treated rats (E) than control rats (D). High protein-induced renin elevation was not affected by COX-2 inhibition with SC-58236 (F).
CoX-2, High Protein, and GFR

Renal cortical CoX-2 and intrarenal renin expression in rats on a high-protein diet. High-protein intake increases GFR and activates the renin-angiotensin system (6, 27, 28). CoX-2 has been proposed to stimulate renin release and biosynthesis under some conditions (12). To investigate whether CoX-2 elevation contributes to activation of the renin-angiotensin system in response to increased dietary protein, intrarenal renin expression was determined by immunostaining and quantified by image analysis. As indicated in Fig. 5, although high-protein intake led to increases in intrarenal renin expression, this increased intrarenal renin expression was not influenced by treatment with the selective CoX-2 inhibitor SC-58236 for a week (fold of control: high-protein diet: 1.75 ± 0.12, P < 0.01 vs. control; high-protein diet + SC-58236: 1.68 ± 0.17, NS vs. high-protein diet; n = 5 in each group).

Discussion

The major findings of this study are 1) renal cortical CoX-2, but not CoX-1, expression correlated with dietary protein intake; 2) renal cortical CoX-2 elevation contributed to high protein-induced GFR elevation; 3) high protein-induced renal cortical CoX-2 elevation was mediated by nNOS; and 4) renal cortical CoX-2 elevation did not appear to be involved in high protein-induced elevations of intrarenal renin expression.

Prostaglandins derived from MD CoX-2 have been proposed to serve as vasodilatory agents for afferent arterioles (12). In cultured rabbit MD/cTALH cells and cultured mouse MD cells, both CoX-2 expression and prostaglandin production are stimulated by low-sodium chloride concentration in the medium (5, 38). We previously showed that rat renal cortical CoX-2 expression is modulated in vivo by maneuvers that affect salt delivery to the MD by altering more proximal NaCl reabsorption, including treatment with acetazolamide or either increased or decreased production of dopamine in the proximal tubule (41). Therefore, renal cortical CoX-2 expression increases in response to reduced luminal NaCl delivery to the MD secondary to increased NaCl reabsorption in the proximal tubule and/or in the loop of Henle.

Increased GFR following amino acid infusion is associated with a reduction in afferent arteriolar resistance (23). Prostaglandin production in kidney cortex increases after high-protein intake (22). Inhibition of prostaglandin production by nonsteroidal anti-inflammatory drugs has been found to abolish the normal increase in GFR after a meat meal or during amino acid infusion (22). Therefore, increased prostaglandin production in kidney cortex may contribute to high protein-induced GFR elevation. High-protein intake causes kidney hypertrophy, particularly in the thick ascending limbs in the inner stripe of the outer medulla (1). Using micropuncture and microperefusion techniques, Seney et al. (1, 31) found that rats on a high-protein diet had higher rates of Na and Cl reabsorption than those on a low-protein diet. In rats on a high-protein diet, increased Na and Cl reabsorption in the loop of Henle and possibly in proximal tubules will lead to reduced luminal fluid NaCl concentration at the level of the MD, which would then be predicted to stimulate CoX-2 expression in MD/cTALH (5, 38, 41). As anticipated, renal cortical CoX-2 expression increased in response to high-protein intake but decreased in response to protein restriction, whereas renal cortical CoX-1 expression was not affected by alterations of dietary protein intake. Therefore, the current studies suggest that renal cortical CoX-2 elevation is responsible for high protein-induced elevation of prostaglandin production in kidney cortex.
inhibition has been reported to enhance afferent arteriolar constriction in response to the activation of TGF induced by benzolamide (9). In dogs, treatment with acetazolamide prevented increases in GFR in response to a meat meal, similar to what was noted with nonsteroidal anti-inflammatory drugs such as aspirin and meclofenamate (22, 33, 36). In the present studies, we found that although high protein-induced kidney hypertrophy was not affected by COX-2 inhibition (Fig. 1), high protein-induced GFR elevation was completely blocked by COX-2 inhibition. Therefore, these studies are consistent with a contribution of renal cortical COX-2 elevation in induction or maintenance of high protein-induced GFR elevation, possibly through dilating afferent arterioles by vasodilatory metabolites such as PGE_{2} and PGI_{2}. As COX-2 is restricted to the MD/cTALH, afferent arteriolar dilation may occur via paracrine regulation. However, we cannot rule out the possibility that vasodilatory prostaglandins may have only a permissive action in high protein-induced GFR elevation.

In addition to prostaglandins, NO derived from nNOS also dilates afferent arterioles (9, 16, 17, 35). Both COX-2 and nNOS are coexpressed in the MD and are stimulated in a number of high-renin states (25). Intrarenal interactions between the COX-2 and nNOS systems have been proposed to play an important role in renal function. NO stimulated prostaglandin production in rabbit kidney (30), and increased COX-2 expression seen in MD/cTALH in response to volume depletion was prevented by inhibition of nNOS (4). NO derived from nNOS has been reported to dilate afferent arterioles directly or indirectly through stimulation of COX-2 activity and COX-2-derived vasodilatory metabolites (9, 16, 17, 35). In the current studies, we determined that a high-protein intake induced both renal cortical nNOS and COX-2 expression, and the increased COX-2 expression was completely blocked by the selective nNOS inhibitor 7-Ni, suggesting that NO may also be a mediator of increased renal cortical COX-2 expression seen in response to a high-protein load. High protein-induced GFR elevation was completely reversed by acute inhibition of nNOS activity with 7-Ni, suggesting that increased nNOS in the MD may contribute to high protein-induced GFR elevation directly through increased NO production and/or indirectly through stimulation of COX-2 activity.

Increased MD COX-2 expression has been detected in other conditions associated with hyperfiltration, including ablation of renal mass and diabetes (10, 18–20, 34), and inhibition of COX-2 activity has been shown to slow progressive glomerulosclerosis in both renal ablation and diabetes mellitus (11, 15, 39), and micropuncture studies demonstrated that protein restriction retarded progressive glomerulosclerosis in both renal ablation and diabetes mellitus (11, 15, 39), and micropuncture studies demonstrated that protein restriction retarded progressive glomerulosclerosis in both renal ablation and diabetes mellitus (11, 15, 39), and micropuncture studies demonstrated that protein restriction retarded progressive glomerulosclerosis in both renal ablation and diabetes mellitus (11, 15, 39), and micropuncture studies demonstrated that protein restriction retarded progressive glomerulosclerosis in both renal ablation and diabetes mellitus (11, 15, 39), and micropuncture studies demonstrated 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