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Tandem antifibrotic actions of L-arginine supplementation and low protein diet during the repair phase of experimental glomerulonephritis

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Tandem antifibrotic actions of L-arginine supplementation and low protein diet during the repair phase of experimental glomerulonephritis.

Background. Based upon the central role transforming growth factor-beta (TGF-β) overexpression appears to play in renal fibrotic diseases, we have recently advocated reduction of TGF-β as a therapeutic target. As part of efforts to determine the strength of this approach, we have undertaken studies to quantitate the effects of currently used and promising therapies in terms of their potential to reduce markers of disease in anti-thymocyte-serum (ATS)-glomerulonephritis in the rat. Here we assess the therapeutic effect of L-arginine supplementation, which has been shown to reduce fibrosis in a number of hypertensive models, given alone or in combination with low protein diet and started 24 hours after disease induction.

Methods. Glomerulonephritis was induced by intravenous injection of OX-7 monoclonal antibody into 200 g Sprague-Dawley rats. Twenty-four hours later animals were placed in groups that were either untreated, treated with 1% L-arginine in drinking water or 6% protein diets or both. On the fifth day of disease 24-hour urine specimens were collected and systemic blood pressure was measured. On the sixth day rats were anesthetized. Kidneys were perfused, tissue was taken for PAS staining and glomeruli were isolated. Aliquots of glomeruli were used for RNA preparation and for culture to determine 72-hour production of TGF-β, fibronectin and plasminogen activator-type 1 (PAI-1), which were assayed by ELISA on culture supernatants. Measures of nitrate and nitrite (NOx) production included plasma NOx, urinary NOx and glomerular production of NOx in culture.

Results. All disease measures except proteinuria and including matrix accumulation, TGF-β, fibronectin and PAI-1 production and mRNA expression for TGF-β, fibronectin and PAI-1 were significantly and similarly reduced by about 50% in groups treated with L-arginine or with low protein diet. Proteinuria was reduced in low protein treated but not in L-arginine supplemented rats. Neither systemic blood pressure nor measures of NO synthesis showed differences between groups that could be attributed to L-arginine supplementation. In contrast, disease-related increases in glomerular production of NOx were markedly reduced by low protein. Combined therapy resulted in small, but statistically significant decreases in most measures of disease.

Conclusions. L-arginine supplementation reduces fibrotic disease in ATS-induced glomerulonephritis if started after disease induction. The absence of evidence for increased NO production related to L-arginine supplementation suggests that L-arginine is acting here through different pathways from those demonstrated in hypertensive models of disease. The data support the ideas that TGF-β reduction is a valid therapeutic target and that quantitation of TGF-β reduction is a useful approach for comparing antifibrotic drug candidates.

Tissue fibrosis results from the accumulation of excessive amounts of pathological extracellular matrix [1]. It is now clear that overexpression of the cytokine transforming growth factor-β (TGF-β) occurs in essentially all fibrotic diseases [1]. A large number of stimuli relevant to renal fibrosis have been shown to cause increased TGF-β expression [reviewed in 2, 3]. It is now conceivable that one or more of these factors, or as yet unidentified factors, lead to increased TGF-β in most, if not all, renal fibrotic diseases. This increased TGF-β directly leads to increased pathological matrix accumulation by: (*1*) increasing synthesis of matrix proteins, (*2*) decreasing degradation of matrix proteins, and (*3*) increasing synthesis and expression on the cell membrane of a group of cell-matrix receptors called integrins [reviewed in 1].

While the interconnections between TGF-β and factors that increase its expression are currently a very active area of research in many laboratories, including our own, we have recently advocated an approach to therapy of renal fibrosis which targets reduction of TGF-β, regardless of the mechanisms involved in its overexpression [2, 3]. This approach is based on the hypothesis that pathways involved in pathological matrix deposition evolved to serve a function critical to evolution, namely, in the face of life-threatening injury, to rapidly stop blood loss and close the wound to avoid sepsis [3]. Such an

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important process might be expected to have many "back-up" pathways, and indeed there is evidence to suggest that this is the case [3]. It may be argued, therefore, that unraveling the interconnections between these pathways will take many years of research, while therapeutic targeting of TGF-β overexpression is feasible now.

There is also ample evidence that the degree of TGF-β overexpression is directly related to the speed with which pathological matrix accumulates. For example, in the streptozotocin model of diabetes where matrix accumulates slowly, TGF-β overexpression increases slowly over many weeks [4]. In anti-thymocyte-serum (ATS)-induced glomerulonephritis, which shows considerable matrix accumulation in six days, TGF-β overexpression occurs rapidly and TGF-β levels are many-fold higher than normal (this study and [5]). In a series of studies of immunohistochemical staining of TGF-β in human renal tissue, TGF-β overexpression was greatest in HIV associated nephropathy, a disease that rapidly progresses to renal failure [6].

Based on these findings, we have undertaken a series of experiments to evaluate current and promising therapies in terms of their ability to reduce pathological overexpression of TGF-β. We have chosen to use the ATS model of glomerulonephritis because it is rapid and highly reproducible and the measures of disease we employ show large differences between normal and diseased glomeruli, a fact that allows small differences in therapeutic efficacy to be seen. In addition, this model of disease has a distinct "injury" phase during which the injected antibody causes complement-mediated lysis of a portion of the glomerular mesangial cells and a distinct "repair" phase, where mesangial cell proliferation, TGF-β overexpression and matrix deposition occur.

Using this model, and studying the repair phase of disease only by starting therapy 24 hours after disease induction, we have recently shown that a greater therapeutic effect is seen with the angiotensin converting enzyme (ACE) inhibitor, enalapril or the angiotensin receptor antagonist, losartan, if doses are higher than those shown to normalize systemic blood pressure in hypertensive rats [5]. Combining these drugs gave no additional therapeutic effect [5]. A preliminary study showed that when maximally effective doses of enalapril are combined with a 6% protein diet, a small additive effect was seen (**Note added in proof**). While these studies give clues as to the mechanisms by which these treatments act, they are designed primarily to give information as to their therapeutic potential with less emphasis on mechanisms. The study presented here looks at the ability of L-arginine supplementation, started 24 hours after disease induction, to reduce TGF-β and other disease markers. L-arginine supplementation was also combined with low protein diet, a therapy whose mechanisms of action are poorly understood, to test for additive effects on disease severity.

L-arginine supplementation is a promising therapy in that it has been shown to reduce histological signs of kidney fibrosis in a number of disease models [7–14] and has been advocated for human trials (abstract, Cianciaruso et al, *J Am Soc Nephrol* 7:1316A, 1996). L-arginine supplementation is also a therapeutic approach where the mechanism of action appears complex [15]. L-arginine is a semi-essential amino acid and a precursor for endogenous production of nitric oxide (NO) [reviewed in 16]. NO normally produced in small amounts by constitutively expressed NO synthases in endothelial cells (eNOS) is an important vasodilator, as demonstrated by studies showing that blockade of NO production with the NOS blocker L-monomethylarginine (L-NAME) leads to increased histological evidence of disease [17]. Dietary L-arginine supplementation is believed to increase eNOS synthesis of NO and thereby to limit hypertensive injury to the kidney. Although it is indisputable that complete blockade of normal endogenous production of NO by endothelial cells is harmful, it is not clear that L-arginine supplementation enhances this production directly through increased availability of substrate. For example, the blood pressure lowering effect of L-arginine infusion is largely mediated through insulin release rather than through increased substrate availability for endothelial NO production [18]. L-arginine is also a substrate for agmatine, a compound whose role in renal disease is just beginning to emerge [reviewed in 19]. Thus, it appears very possible that the therapeutic action of L-arginine supplementation is through pathways other than simply enhanced vasodilatory NO production by endothelial cells.

Another complicating factor is that tissue injury is dependent upon generation of cytotoxic quantities of NO through induction of another isoform of nitric oxide synthase, inducible NOS (iNOS) in a number of models of disease, including lupus nephritis and kidney ischemia-reperfusion [20–22]. Increased expression of iNOS in patients with IgA nephropathy and renal transplant rejection has been shown (abstract, Ikegaya et al, *J Am Soc Nephrol* 7:1563A, 1996) [23]. Renal transplant rejection is also paralleled by increased NO in the urine [24]. Inducible NOS induction and NO generation are also involved in the injury phase of ATS-induced disease where restriction of L-arginine intake before disease induction and pretreatment with the NOS blocker L-NAME significantly reduce cell lysis [20]. In contrast, dietary supplementation of L-arginine, given only during the injury phase of disease, increases injury and the fibrotic response that follows [25].

Dietary manipulations of L-arginine therefore exemplify the mechanistic complexity of promising therapies and help to make the argument that empirical data on therapeutic efficacy have value.

METHODS

Materials

If not otherwise indicated, materials, chemicals or culture media were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

The studies were performed in male Sprague-Dawley (SD) rats (200 to 270 g) obtained from Sasco (Omaha, NB, USA). Glomerulonephritis was induced by tail vein injection of the monoclonal anti-Thy 1.1 antibody OX-7 (1.5 mg/kg). OX-7 binds to a Thy 1-like epitope on the surface of mesangial cells and causes complement-dependent cell lysis [26]. Normal control animals were injected with the same volume of phosphate-buffered saline (PBS). Body weight, food and water intake were monitored individually at the beginning and end of each experiment.

OX-7 production

The monoclonal antibody was produced in the ascitic fluid of primed adult female BALB/c mice (Charles River, Wilmington, MA, USA). The hybridoma was obtained from American Type Culture Collection (Rockville, MD, USA) and the cells were grown in RPMI 1640 (Gibco BRL, Long Island, NY, USA) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin and 25 mmol/L HEPES buffer at 37°C/5% CO_2 and passaged at confluence. The mice were primed with two intraperitoneal injections of 0.5 ml pristane (2,6,10,14-tetramethyldecanoic acid) one week apart. Three days after the second pristane administration, the hybridoma cells were spun at 3000 *g* for five minutes and resuspended in PBS (pH 7.4). Approximately 1 to 2 million cells in 0.5 ml PBS were injected intraperitoneally per mouse. When the mice developed noticeable ascites, they were anesthetized with ether and the ascitic fluid was collected.

To purify the IgG fraction, ascitic fluid was combined with 2/10 volumes 1.0 mol/L Tris (pH 8.0) and applied to a pre-equilibrated Protein G column (Pierce, Rockford, IL, USA). The antibody fraction was eluted with 100 mmol/L glycine (pH 3.0) and promptly neutralized with Tris (pH 9.0). After extensive dialysis against PBS (pH 7.4) the antibody concentration was adjusted to 1 mg/mL by reading the adsorbance at 280 nm and stored at −70°C until use.

Experimental design

For at least three days before induction of glomerulonephritis, animals were fed a normal protein diet (22% casein, Teklad No. 86 550; Teklad Premier Laboratory Diets, Madison, WI, USA) with an L-arginine content of 0.85%. Twenty-four hours after OX-7 or PBS-injection, the rats ($N = 8$ per group) were assigned to the following dietary modifications: (*1*) normal control animals on a normal protein diet; (*2*) OX-7-injected animals (GN) on a normal protein diet (GN-NP); (*3*) GN rats on a normal protein diet with 1% L-arginine in the drinking water (GN-NP + Arg); (*4*) GN rats on a low protein diet (6% casein, Teklad No. 86 551) (GN-LP); and (*5*) GN rats on a low protein diet with 1% L-arginine in the drinking water (GN-LP + Arg). The normal protein and the low protein diet were identical in fat and mineral content. The low protein diet was made isocaloric (14.6 J/g) by addition of sucrose.

Five days after disease induction, systolic blood pressure was measured in the conscious state by tail cuff [27]. The rats were housed in metabolic cages and urine was collected for 24 hours. Six days after injection, the animals were anesthetized with ether. Following a midline abdominal incision, heparinized blood was drawn from the lower abdominal aorta. The blood was immediately centrifuged and plasma was frozen at −70°C until analysis of plasma L-arginine and nitrite + nitrate (NOx) levels. The kidneys were subsequently perfused with 30 mL ice-cold PBS and harvested. Cortical tissue was fixed in 10% neutral buffered formalin for histological examination. Glomeruli from individual rats were isolated by a graded sieving technique (150, 125, 106 and 75 μm mesh metal sieves) as described previously [28].

An aliquot of glomeruli was resuspended in DMEM supplemented with 0.1 U/mL insulin, 100 U/mL penicillin, 100 μg/mL streptomycin and 25 mmol/L HEPES buffer. After 72 hours of incubation at 37°C/5% CO_2 the supernatant was harvested and stored at −70°C until analysis of glomerular TGF-β1, fibronectin and PAI-1 and NO production. Preliminary studies have indicated that production of TGF-β1, fibronectin, PAI-1 and NO by isolated glomeruli are constant for 72 hours of culture (unpublished observations). The 72 hour harvest of culture supernatant was chosen based on these data and on the fact that quantities of these molecules are sufficient at this time to obtain accurate ELISA measurements. The remaining glomeruli were lyzed in Trizol™ Reagent and homogenized with three short bursts of a tissue homogenizer. After five minutes of incubation at room temperature, the samples were stored at −70°C until RNA isolation.

Light microscopy

Microscopic examinations were performed in a blinded fashion on 3 μm sections of paraffin-embedded tissues stained with periodic acid Schiff (PAS). Glomerular matrix expansion was evaluated in 30 glomeruli from each rat by scoring the percentage of mesangial matrix occupying each glomerulus where: 0 = 0%, 1 = 25%, 2 = 50%, 3 = 75% and 4 = 100%.

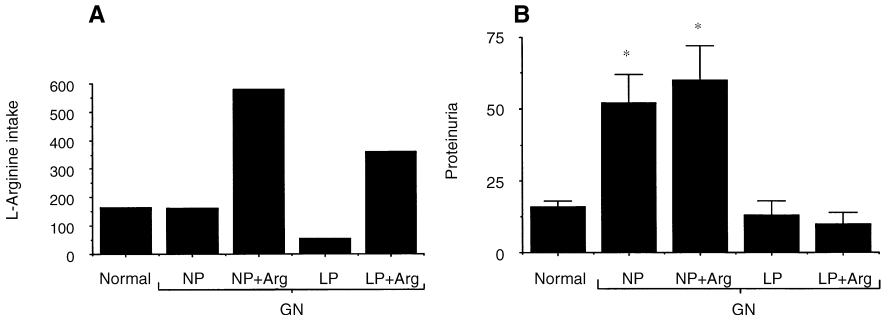

Fig. 1. (*A*) Mean L-arginine intake over the course of the experiment. (*B*) Urinary protein excretion measured on urine collected for the 24 hours preceding sacrifice. After induction of ATS glomerulonephritis (GN), rats were fed a normal protein diet (NP, 22% casein) or a low protein diet (LP, 6% casein). In some groups the drinking water supplemented 1% L-arginine (+Arg).

TGF-β1, fibronectin and PAI-1 ELISAs

TGF-β1 production of cultured glomeruli was measured after acid-activation using a commercially available kit (Quantikine™; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Fibronectin and PAI-1 synthesis were measured with modified inhibitory enzyme-linked immunosorbant assays (ELISA) according to published methods [29]. Three samples from each rat were analyzed.

RNA preparation and Northern hybridization

Total RNA was extracted by a guanidinium isothiocyanate method using Trizol™ Reagent according to the manufacturer's instructions. Pooled glomeruli were used for subsequent RNA extraction. For Northern analysis, RNA was denatured and fractionated by electrophoresis through a 1.8% agarose gel (20 μg/lane) and transferred to a Hybond nylon membrane (Amersham Corp., UK). Nucleic acids were immobilized by UV irradiation. Membranes were prehybridized with 50% formamide, 10% Denhardt's solution, 0.1% SDS, 5 × SSC and 200 μg/ml denatured salmon sperm DNA and hybridized with DNA probes labeled with ^{32}P-dCTP by random oligonucleotide priming (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). The blots were washed in 2 × SSC, 0.1% SDS at room temperature for 15 minutes and in 0.1 × SSC, 0.1% SDS at 55°C for 30 minutes. DNA probes used were: (*1*) rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (a gift from Drs. P. Kondaiah and M.B. Sporn) [30], (*2*) TGF-β1 cDNA (kindly provided from Dr. D.L. Moses) [31], (*3*) fibronectin-EDA cDNA (a generous gift from Dr. R.O. Hynes) [32], (*4*) PAI-1 cDNA (kindly provided by Dr. T.D. Gelehrter) [33]. Three blots per probe were performed.

Proteinuria

Proteinuria was measured using the Bradford method and is expressed as mg protein/24 hours [28].

NOx measurements

Nitrite and nitrate are stable endproducts of NO and served as indicators of NO synthesis. Plasma and urinary NO_2/NO_3 (NOx) levels were measured by the Griess reaction after nitrate reductase treatment [34]. Briefly, samples were diluted in PBS and incubated at room temperature in the presence of 0.05 μmol/L NADPH and 0.05 Units nitrate reductase (Boehringer Mannheim Biochemicals). After 45 minutes incubation at room temperature, proteins were precipitated with 70% zinc sulfate solution and samples were centrifuged at 14,000 *g* for five minutes. One hundred microliters of sample was then mixed with 100 μL Griess reagent (0.05% N-(1-naphthyl) ethylene diamine dihydrochloride, 0.5% sulfanilamide in 45% glacial acetic acid) in 96 well plates. After 10 minutes incubation in the dark, adsorbance was read at 546 nm in an automated plate reader (Thermomax: Molecular Devices, Menlo Park, CA, USA). Standard samples were prepared with sodium nitrate and were also nitrate reductase-treated.

For measurement of the glomerular NO production, 100 μL of samples were mixed with 100 μL of Griess reagent. Standard probes were prepared using sodium nitrite.

L-arginine level analysis

Plasma L-arginine levels were determined by an automated amino acid analyzer [35].

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis between the groups was performed by ANOVA and subsequent *t*-testing with Bonferroni correction for multiple comparison. A P value < 0.05 was considered significant.

RESULTS

Body weight, food and water intake and L-arginine intake

There were no significant differences between the groups in body weight or food and water intake during the experiment. Systemic blood pressures also did not vary and were as follows: mean ± SEM; normal control, 111 ± 8 mm Hg; GN-NP, 110 ± 6 mm Hg; GN-NP + Arg, 113 ± 3 mm Hg; GN-LP, 114 ± 56 mm Hg; GN-LP + Arg,

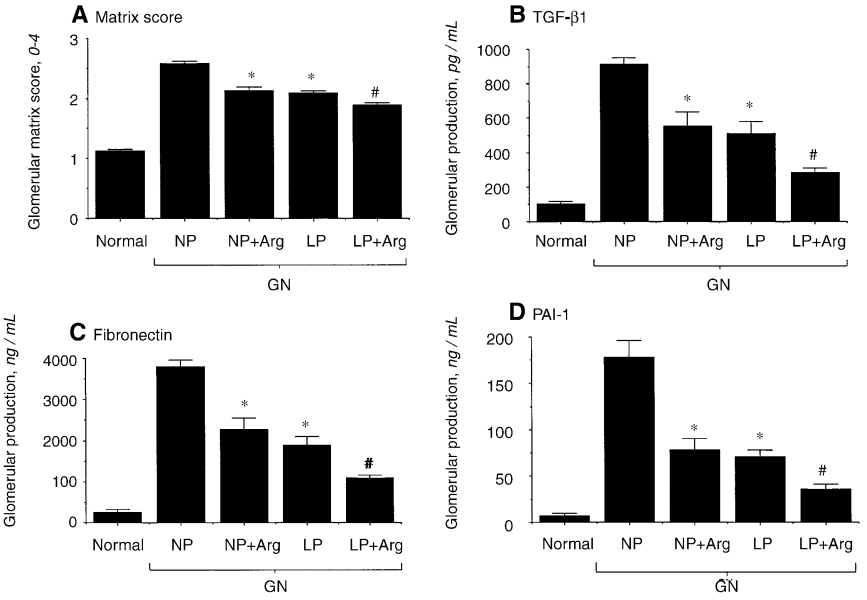

Fig. 2. (*A*) Glomerular matrix accumulation six days after induction of ATS glomerulonephritis (GN). Animals were fed a normal protein diet (NP, 22% casein) or a low protein diet (LP, 6% casein). In some groups the drinking water supplemented 1% L-arginine (+Arg). *B*, *C* and *D* show the glomerular production of TGF-β1, fibronectin and PAI-1 in supernatants of cultured glomeruli, respectively. $*P < 0.01$ versus GN-NP, $\#P < 0.05$ versus GN-NP + ARG and GN-LP.

111 ± 7 mm Hg. Figure 1A shows L-arginine intake. Because L-arginine intake was determined by weighing food, the data are estimates and measures of variability are not given. L-arginine feeding increased L-arginine intake 3.6-fold above normal in rats on a normal protein diet and 7.7-fold above normal in rats on a low protein diet. Compared to rats fed a normal protein diet, L-arginine intake was 3.5-fold lower in the low protein diet group drinking tap water only (Fig. 1A).

Proteinuria

Proteinuria data are shown in Figure 1B. Proteinuria was significantly increased in nephritic animals fed a normal protein diet whether they were supplementated with L-arginine or not (normal control, 16 ± 2 mg/24 h; GN-NP, 52 ± 10 mg/24 h; GN-NP + Arg, 60 ± 12 mg/24 h, $P < 0.01$). Proteinuria in nephritic rats fed a low protein diet was significantly reduced compared to nephritic rats fed a normal protein diet, again whether they were supplemented with L-arginine or not (GN-NP, 52 ± 10 mg/24 h, GN-LP, 13 ± 5 mg/24 h; GN-LP + Arg, 10 ± 4 mg/24 h; $P < 0.01$). In addition, proteinuria was slightly, but not significantly reduced in nephritic rats fed low protein compared to normal rats (Fig. 1B). Thus, in nephritic rats, proteinuria followed protein intake and not L-arginine intake.

Effect of L-arginine supplementation and low protein feeding on markers of fibrotic disease 6 days after disease induction

Histological examination. As shown graphically in Figure 2A, glomerular matrix accumulation was significantly and to a similar extent reduced by both supplementation of the drinking water with 1% L-arginine and dietary protein restriction (normal control, 2.58 ± 0.04; GN-NP + Arg, 2.13 ± 0.06; GN-LP, 2.09 ± 0.04).

Glomerular production of TGF-β1, fibronectin and PAI-1. Glomerular TGF-β1 protein production in vitro was increased by 8.9-fold in untreated nephritic rats (GN-NP) compared to the normal controls (Fig. 2B). Glomerular fibronectin synthesis was elevated 15-fold and glomerular PAI-1 synthesis 25-fold (Fig. 2 C, D, respectively). A significant reduction in glomerular production of TGF-β, fibronectin and PAI-1 was seen in the rats treated with either L-arginine supplementation or low protein diet (Fig. 2 B–D). Compared to the disease control animals, L-arginine administration reduced glomerular production of TGF-β1 by 40%, fibronectin by 41% and PAI-1 by 50% (GN-NP + Arg). Low protein feeding decreased production of TGF-β1, fibronectin and PAI-1 protein by 44%, 50% and 60% (GN-LP). The extent of reduction in pathological overexpression of TGF-β, fibronectin and PAI-1 did not differ between the two single therapies, L-arginine supplementation and low protein diet ($P > 0.05$).

Glomerular expression of mRNAs for TGF-β1, fibronectin and PAI-1. A representative Northern blot is shown in Figure 3A. Expression of mRNA, quantitated by laser densitometry and averaged for three blots, is shown graphically in Figures 3B–D. Disease alone (GN-NP) resulted in increases of 3.7-, 34- and 62-fold for TGF-β1, fibronectin and PAI-1 mRNAs (Fig. 3 B–D, respectively). A significant reduction in mRNA expression was seen in the rats treated with either L-arginine supplementation (GN-NP + Arg) or low protein diet (GN-LP) (Fig. 2A–D and 3A–D). Compared to the disease control

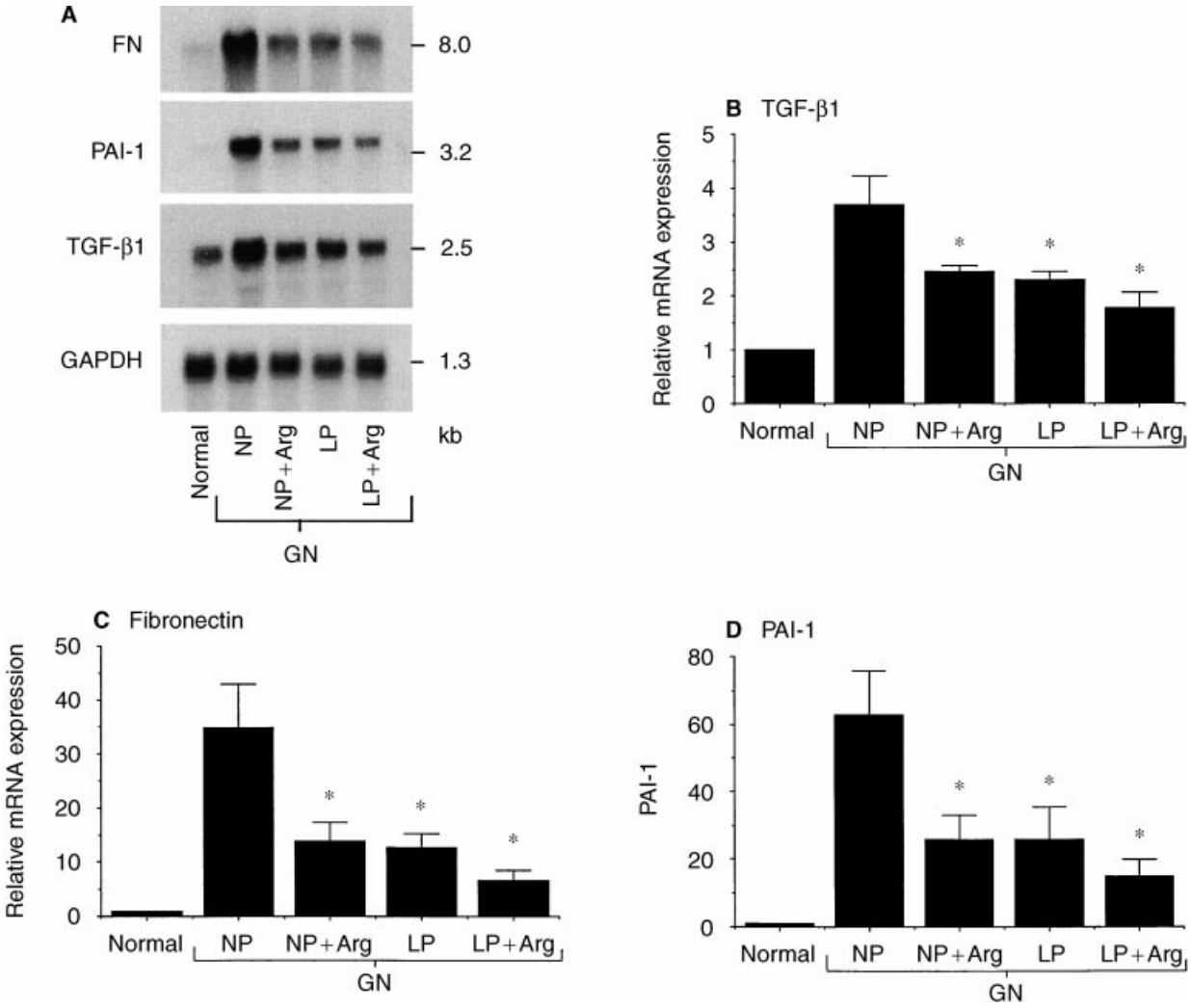

Fig. 3. (*A*) A representative Northern blot showing glomerular mRNA expression of GAPDH, TGF-β1, fibronectin and PAI-1 harvested six days after induction of glomerulonephritis (GN). After correction for quantities of mRNA for GAPDH, mRNA levels are shown graphically for (*B*) TGF-β1, (*C*) fibronectin and (*D*) PAI-1. Molecular size markers are shown on the right. *$P < 0.05$ versus GN-NP.

animals (GN-NP), L-arginine administration reduced glomerular mRNA of TGF-β1 by 34%, fibronectin by 62% and PAI-1 by 59% (GN-NP + Arg; Fig. 3B–D). Low protein feeding decreased mRNA expression of TGF-β1, fibronectin and PAI-1 by 38%, 64% and 58%, respectively (GN-LP; Fig. 3B–D). Again, single therapy resulted in very similar reductions in mRNA expression.

Additive effects of L-arginine supplementation and low protein diet. Using as powerful measures of disease severity matrix score, glomerular production of TGF-β, fibronectin and PAI-1 and Northern blotting of RNA to quantitate the mRNAs for these proteins, a significantly enhanced therapeutic effect was seen when both therapies were combined (Figs. 2 and 3). Dual therapy reduced all measures of disease, except proteinuria (Fig. 1B), to a greater extent than either therapy alone. As shown in Figure 2, a significant reduction in matrix score and glomerular production of TGF-β, fibronectin and PAI-1 was seen with combined therapy. TGF-β overproduction was reduced a total of 69%, fibronectin overproduction by 71% and PAI-1 overproduction by 79% (Fig. 2). The reductions in TGF-β1, fibronectin and PAI-1 mRNA levels seen with dual therapy were 52%, 81% and 77%, respectively (Fig. 3).

Effects of L-arginine supplementation and low protein diet on plasma L-arginine levels. Arterial L-arginine levels are shown in Figure 4A. Disease itself (GN-NP) resulted in significantly decreased arterial L-arginine levels whether rats were fed normal (NP) or low (LP) protein diet, suggesting either increased utilization of L-arginine during disease or decreased synthesis by proximal tubular cells that are the main site of in vivo L-arginine production [36]. L-arginine supplementation corrected this disease-induced decrease and gave plasma values 338%

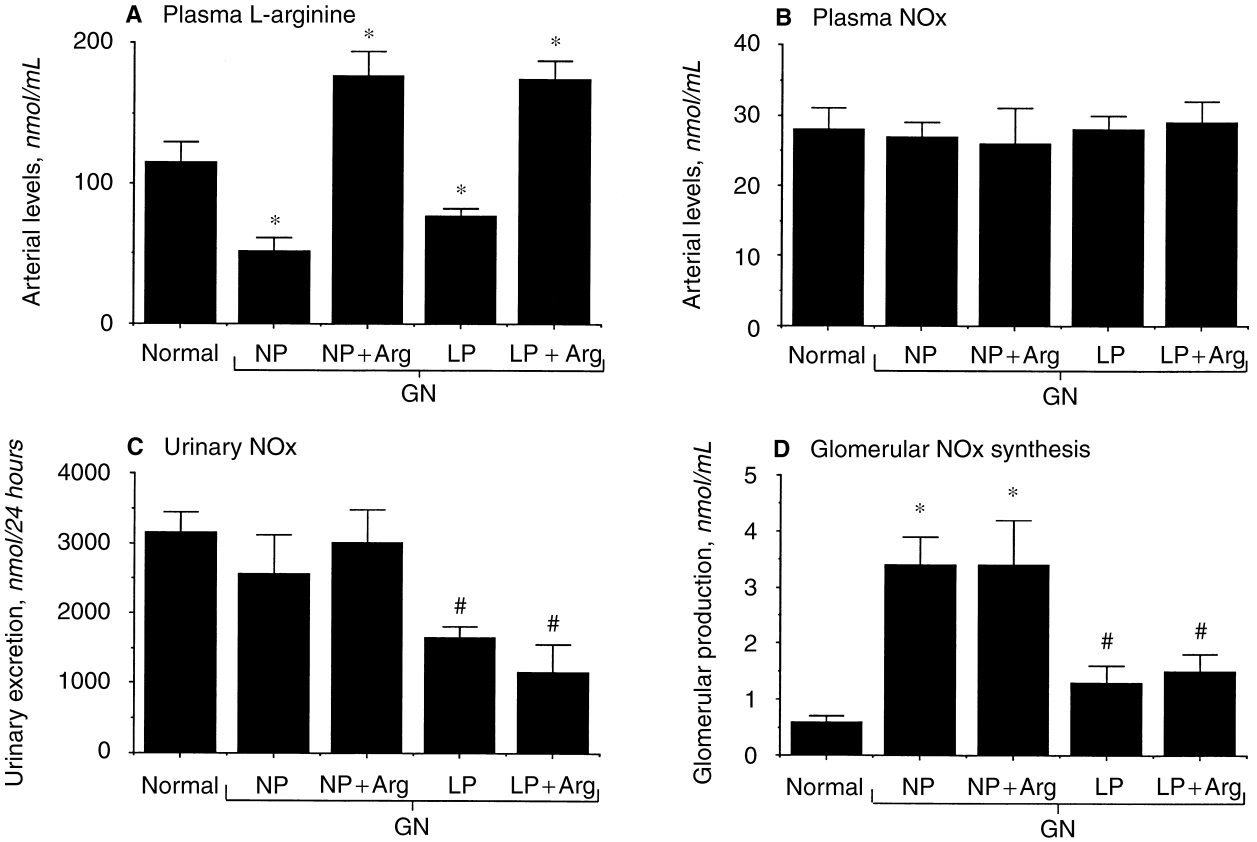

Fig. 4. Arterial L-arginine concentrations (*A*) and NOx levels (*B*) six days after disease induction. Urinary NOx excretion over the 24 hours before sacrifice and glomerular NOx production in culture over 72 hours after harvest are shown in (*C*) and (*D*), respectively. $*P < 0.05$ versus normal, $\#P < 0.05$ versus normal, GN-NP and GN-NP + Arg.

and 226% above normal levels in diseased animals on normal and low protein diet, respectively.

Effects of L-arginine supplementation and low protein diet on nitrite + nitrate (NOx) content of plasma and urine and NOx production by glomeruli in culture

NOx was measured in three types of samples, plasma (Fig. 4B), urine (Fig. 4C) and supernatant from glomeruli cultured for 72 hours (Fig. 4D). A striking feature of the data shown in these figures is that the level of NOx in no case reflected the presence of absence of L-arginine supplementation. Where there were differences between treatment groups, they followed protein intake. Thus, urinary excretion of NOx was normal in nephritic animals on a normal protein diet and significantly reduced in both groups of animals on a low protein diet. Arterial NOx levels were the same in all groups, suggesting that L-arginine supplementation does not cause large, systemic changes in NO production (Fig. 4B). Urinary NOx excretion was also very similar in normal, untreated nephritic and nephritic rats on normal protein diets with L-arginine supplementation, suggesting that L-arginine supplementation alone does not increase urinary excretion of NOx (Fig. 4C). Feeding nephritic rats a low protein diet, whether supplemented with L-arginine or not, lead to a dramatic decrease in urinary NOx excretion (Fig. 4C). The finding that plasma NOx levels are not reduced by low protein feeding (Fig. 4B) suggests that the decrease in urinary NOx (Fig. 4C) is not due to systemic decreases in NO production, but may be due to decreased production by the nephron of nephritic rats on a low protein diet. However, glomerular production of NOx in these rats is significantly elevated compared to normal glomeruli (Fig. 4D). Thus, it is likely the reduction of urinary excretion of NOx in nephritic animals fed a low protein diet is due to decreased production by proximal tubular cells.

Also shown in Figure 4D are data indicating that nephritic rat glomeruli on a normal protein diet, whether supplemented with L-arginine or not, produce significantly more NOx than normal control glomeruli, a difference that is not reflected in urinary NOx (Fig. 4C). Based on the data in Figure 4, it appears that: (*1*) plasma L-arginine levels are reduced by disease itself both in the presence of normal and high L-arginine intake. (*2*) Neither the presence nor absence of ATS-induced glo-

merulonephritis or large differences in L-arginine intake (Fig. 1), plasma L-arginine levels or protein intake produce detectable changes in plasma NOx content. (*3*) Neither plasma L-arginine levels nor disease state are clearly reflected in urinary NOx content, but appear to be greatly affected by low protein intake. (*4*) Glomerular NO production is about threefold increased by ATS-GN and is not altered by L-arginine supplementation but is greatly reduced by low protein intake. (*5*) Differences in glomerular NOx synthesis are not clearly reflected by urinary NOx content. These data reveal a clear effect of low protein intake on NO synthesis by the nephron, an interesting, and we believe, a new link between NO and low protein intake.

DISCUSSION

The present study demonstrates the power of the measures employed to compare different therapies side-by-side. For reasons covered in the Introduction, TGF-β was used as a key marker of disease. The other major markers were fibronectin and PAI-1; both strongly induced by TGF-β1 in vitro [37, 38]. Fibronectin expression was used as a sensitive marker of extracellular matrix synthesis. The protease inhibitor PAI-1 was used as an indicator of the activity of matrix degrading systems. The importance of PAI-1 in matrix breakdown has recently been shown in a study of mesangial cells in culture [39] as well as in an in vivo model of lung fibrosis [40]. Following bleomycin administration, mice with genetic PAI-1 overexpression showed markedly increased collagen deposition, while the fibrotic response was greatly diminished in mice with a disrupted PAI-1 gene [40]. Using these disease markers in the present study, increases in glomerular production of 8.9-fold for TGF-β1, 15-fold for fibronectin and 25-fold for PAI-1 were seen in untreated diseased rats compared to normal controls. These substantial differences between normal and untreated diseased rats provide a large therapeutic window that enables detection of small differences in drug efficacy. This is demonstrated by the small, but clear and statistically significant additive therapeutic effect seen with L-arginine supplementation and low protein intake. It should be pointed out that the mRNA data shown in Figure 3 were decreased in the dual therapy group compared to the single therapy groups, although this difference did not achieve statistical significance. This may simply be due to the greater sample size (8 vs. 3) for the ELISA measurements. On the other hand, we have obtained similar results in a number of studies of this kind, suggesting that the ELISA determinations are more sensitive than the Northern data [5, 25]. Whatever the reason for these findings, we regard production of proteins as more relevant to fibrosis than expression of their mRNAs.

All disease measures except proteinuria were significantly reduced by L-arginine supplementation, confirming other studies, mostly in hypertensive models of disease, showing that this therapeutic approach is potentially of benefit to humans. The fact that proteinuria was not reduced by L-arginine supplementation, while all other measures of disease were decreased is interesting, although the reasons why proteinuria did not follow fibrotic disease severity are unclear. It may be relevant to point out that proteinuria in antithymocyte serum-induced glomerulonephritis does not always follow the severity of the fibrotic response. Some rat strains do not show proteinuria as part of this disease [41]. In addition, using the same rat strain, the degree of proteinuria varies with the antibody or antiserum used to produce disease far more than the fibrotic response (unpublished observation). Nonetheless, in these Sprague-Dawley rats with OX-7 antibody, proteinuria at day 6 usually follows severity of fibrosis. Therefore, the finding that proteinuria is not reduced by L-arginine supplementation is of interest.

Although this study provides another example of the antifibrotic effects of dietary L-arginine supplementation, it remains unclear which metabolite(s) or pathway(s) mediate this effect. Studies using subtotal nephrectomy, ureteral obstruction and salt sensitive Dahl/Rapp rats have suggested that L-arginine supplementation increases endothelial NO production, thereby limiting hypertensive renal tissue damage [8, 11, 13]. This view is supported by evidence for increased NO synthesis in these models following L-arginine administration. In ureteral obstruction and in salt-sensitive Dahl-Rapp rats, L-arginine administration also reduced arterial pressure [9, 13]. In rats with 5/6 nephrectomy, L-arginine supplementation normalized glomerular pressure [8]. However, in the ATS model of glomerulonephritis studied here, no blood pressure elevation was seen and, although L-arginine supplementation significantly increased arterial L-arginine levels it had no effect on systemic blood pressure and no effect on measures of NO. Therefore, we found no evidence that this therapy, in this model, acts through enhanced systemic or glomerular NO production.

As pointed out in a recent review article [42], there is still much to learn about the meaning of urinary and plasma NOx measurements. Even with this in mind, it appears likely that L-arginine supplementation reduces fibrotic disease by other mechanisms and not solely through enhanced vasodilatory NO production by endothelial cells. Taking the data presented here with other data in hypertensive diseases, it appears that L-arginine acts through more than one pathway. If this were the case, one would predict that if disease reduction with L-arginine supplementation were quantified in a hypertensive model of renal fibrosis, as we have done here in ATS-nephritis with small, if any hypertensive component [43],

reduction of TGF-β overexpression might be greater than the approximately 50% observed in this study.

The data on animals fed low protein confirm earlier demonstrations of the effects of 6% protein diet on reduction of fibrotic disease in this model [27]. In addition it quantitates the therapeutic effect, making comparison with other therapies possible. Further, as discussed above, the data reveals a fascinating link between low protein intake and reduction in renal NO synthesis. It is unlikely that the mechanism of this is the low L-arginine content of low protein diet leading to decreased L-arginine as substrate for cytotoxic NO production by iNOS because glomerular NO production was substantially reduced in animals given low protein and L-arginine as well as those treated with low protein diet alone (Fig. 4D).

The present study suggests that the antifibrotic effect of L-arginine supplementation is mediated through a reduction in TGF-β overexpression. L-arginine administration significantly reduced glomerular expression of TGF-β and the matrix constituents fibronectin and PAI-1. Where it has been investigated, reduction of pathological matrix deposition has generally followed reduction in overexpression of TGF-β. One exception is a study where rats with unilateral ureteral obstruction were given drinking water supplemented with 1% L-arginine [11]. The authors suggest that direct effects of NO on collagen synthesis may be responsible for this unusual result [44, 45]. The data presented here with ATS-nephritis give no suggestion of direct effects of L-arginine on matrix synthesis in that matrix deposition and all other measures of disease except proteinuria closely follow TGF-β.

The study presented here is the second in a series designed to quantitate the efficacy of current and promising therapies in the ATS model of glomerulonephritis where reduction of TGF-β overexpression is the target. Although many publications using animal models and human subjects have shown associations between reductions in TGF-β and disease, few have been quantitative [reviewed in 2]. A major impetus for this quantitative approach came from a review of the literature on the TGF-β reducing effects of drugs designed to block angiotensin II. A striking finding was that most studies, regardless of the disease model, the drug or the dose used, achieved reductions in TGF-β overexpression of approximately 50% [2]. This stimulated us to see whether this was the case if multiple drugs and/or multiple doses were compared side-by-side in the repair phase of ATS glomerulonephritis. It is very interesting that our work to date using this approach seems to indicate similar maximal therapeutic effects regardless of the therapy used. Thus, maximally effective doses of enalapril or losartan, 6% protein diet or 1% L-arginine supplementation all achieved reductions in markers of fibrotic disease at day 6 in the range of 50% [5].

If one accepts the idea that TGF-β overexpression is caused by multiple pathological stimuli acting even within one disease and that reduction of TGF-β overexpression is currently our best therapeutic target, these data clearly point to therapeutic approaches involving drug combinations. In this regard, the absence of additive effects with enalapril and losartan combined [5] and the small additive effects seen here with L-arginine and low protein and elsewhere with enalapril and low protein (**Note added in proof**) are of interest. Finally, these studies suggest that therapies that more directly target TGF-β overexpression, such as antibodies, TGF-β receptor antagonists or the natural TGF-β inhibitor, decorin, may be required to halt the progression of renal fibrosis. It should be noted that while there is considerable evidence that TGF-β overexpression currently appears to be our best therapeutic target, reduction of this cytokine to subnormal levels may well be deleterious because of the many beneficial roles of TGF-β in tissue repair.

In conclusion, the present study shows that L-arginine supplementation is therapeutic in ATS-glomerulonephritis if started after the injury phase of disease. The data suggest that in this disease model the mechanism underlying this effect is not increased generation of NO or reductions in systemic blood pressure as has been suggested by findings in hypertensive disease models. Therefore, L-arginine may act through multiple pathways to reduce fibrosis. Since L-arginine supplementation may increase tissue injury when iNOS is involved [25], only further studies will reveal the true therapeutic potential of L-arginine supplementation in humans.

Note added in proof

I Peters H, Border WA, Noble NA: Angiotensin II blockade and low-protein diet produce additive therapeutic effects in experimental glomerulonephritis. *Kidney Int* (in press)

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