Amino acids induce indicators of response to injury in glomerular mesangial cells

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—High-protein diets exacerbate glomerular hyperfiltration and the progression of diabetic nephropathy. The purpose of this study was to determine whether amino acids also produce nonhemodynamic injury in the glomerulus. When rat mesangial cells were cultured with an amino acid mixture designed to replicate the composition in plasma after protein feeding, production of mRNA (Northern blot analysis) and/or protein (ELISA or Western blot analysis) for transforming growth factor-β1 (TGF-β1), fibronectin, thrombospondin-1 (TSP-1), and collagen IV were enhanced in a manner comparable to a culture with high glucose (30.5 mM). The bioactive portion of total TGF-β (NRK assay) increased in response to amino acids. The TSP-1 antagonist LSKL peptide reduced bioactive TGF-β and fibronectin, indicating the dependence of TGF-β bioactivity on TSP-1. DNA synthesis ([3H]thymidine incorporation), an index of cellular proliferation, increased in response to amino acids and was further enhanced by culture with increased levels of both amino acids and glucose. TGF-β1 and matrix proteins increased when mesangial cells were cultured with excess L-arginine (2.08 mM) alone. Although L-arginine is the precursor of nitric oxide (NO), such responses to amino acids do not appear to be mediated through increased NO production. NO metabolites decreased in the media, and these responses to mixed amino acids or L-arginine were not prevented by NO synthase inhibition. In conclusion, amino acids induce indicators of response to injury in mesangial cells, even when hemodynamic stress is absent. In conditions associated with increased circulating amino acids, such as diabetes and/or a high-protein diet, direct cellular effects could contribute to glomerular injury.

transforming growth factor-β; thrombospondin-1; extracellular matrix proteins; mesangial cell proliferation; hyperglycemia

HIGH-PROTEIN DIETS, RESULTING in increased circulating amino acids, produce glomerular hyperfiltration and hypertension, major mechanisms of progressive renal injury (5, 47). In the clinical setting of diabetes, this is a particular concern because high-protein diets have been promoted to modulate hyperglycemia or to promote weight loss. Furthermore, poor glycemic control also increases plasma amino acid concentrations (10). As such, hyperaminoacidemia is common in diabetes and could exacerbate renal injury. The glomerular hyperfiltration associated with increased circulating amino acids is similar to that induced by hyperglycemia (5, 47). In clinical studies, we have demonstrated that people with poorly controlled type 1 or type 2 diabetes have an augmented glomerular hyperfiltration response to amino acids, which can be corrected by strict glycemic control (41–43). The mechanism(s) of the interaction between hyperglycemia and amino acids is unknown. Hormonal mediators have been proposed, but none has been identified (43). Whether amino acids have direct cellular effects that enhance or cause injury is unexplored.

The mesangial cell is a key cell involved in the control of glomerular hemodynamics, as well as in the response to injury. Recognized causes of injury, such as hyperglycemia, have both hemodynamic and direct cellular effects. In vitro models utilizing mesangial cell culture have shown that cellular stretch (a model of glomerular hypertension) or high-glucose media (a model of hyperglycemia) induce production of transforming growth factor-β (TGF-β) (19, 31, 48). TGF-β is a profibrotic cytokine that is a seminal cellular mediator of response to injury (36). TGF-β is secreted in an inactive form, which can be activated by diverse mechanisms, including interactions with the matrix protein thrombospondin-1 (TSP-1) (20). In mesangial cells cultured with high-glucose media, TSP-1 expression is also elevated and enhances TGF-β bioactivity (28, 39). The potential influence of amino acids on these processes has not been evaluated.

Either a mixture or certain individual amino acids could have renal effects. L-Arginine is a plausible candidate because it is the precursor of nitric oxide (NO). NO has many important functions in the kidney, in-
including promoting vasodilation, which may enhance glomerular hyperfiltration, as well as direct cellular effects, which may be protective or injurious. Depending on the experimental system, increased NO has been variably reported to either decrease or increase TGF-β production and fibrosis (9, 21, 37). In mesangial cells cultured with a high-glucose concentration, stimulation of NO production through endogenous (interleukin-1β) or exogenous (NO donor) methods prevented TGF-β induction (9). Furthermore, suppression of NO by hyperglycemia has been reported to increase TGF-β activation and mesangial matrix production (40, 44). In mesangial cells, it is unclear to what extent L-arginine functions as an NO precursor without cytokine stimulation of inducible NO synthase, and possible relationships among amino acids, NO, and TGF-β have not been studied.

The overall goal of this study was to determine whether amino acids, in the absence of hemodynamic stress, produce mesangial cell responses associated with the type of glomerular injury observed in diabetes. A mixture of amino acids, designed to replicate the composition in plasma after protein feeding, was evaluated for effects on expression of TGF-β1 and matrix proteins as well as on mesangial cell proliferation and viability (other indicators of response to injury). These effects were explored with and without a concomitant high-glucose concentration. Whether TSP-1 influences amino acid-induced TGF-β bioactivity was assessed by use of an inhibitory peptide. The effects of L-arginine on NO and expression of TGF-β1 and matrix proteins were also determined.

METHODS

Mesangial Cell Isolation and Culture

Rat mesangial cells were recovered from glomeruli isolated by sieving the cortex of kidneys excised from 6-mo-old female Sprague-Dawley rats. Mesangial cells were grown in DMEM (Life Technologies, Gaithersburg, MD), supplemented with penicillin-streptomycin (100 U/ml) and 10% heat-inactivated fetal bovine serum (Summit Technologies, Ft. Collins, CO) in a humified atmosphere of 5% CO2 at 37°C. Cells were passaged at confluence using 0.025% trypsin (Life Technologies) and used for experiments between passages 4–6 and 14–19.

Experimental Conditions

Increased levels of amino acids and glucose. Mesangial cells were seeded into 100-mm dishes or 24-well plates (Nunclon, Cambridge, MA) at 10,000 cells/cm2. When cells reached confluence, they were made quiescent in serum-free DMEM for 48 h. Cells were then exposed to experimental conditions for 48 h (Table 1): 1) control, serum-free DMEM; 2) control plus LSKL peptide (5 μM); 3) control plus SLLK peptide (5 μM); 4) increased amino acids (Table 1); 5) increased amino acids plus LSKL peptide (5 μM); and 6) increased amino acids plus SLLK peptide (5 μM). The conditioned media were removed and 2 μg/ml each of the protease inhibitors pepstatin, leupeptin, antipain, and aprotinin (Sigma, St. Louis, MO) were added. The media were then stored at −70°C until assayed for TGF-β bioactivity and fibronectin protein.

Increased level of L-arginine and NO synthase inhibition. Confluent mesangial cells were made quiescent in serum-free DMEM for 48 h. Cells were then placed in experimental conditions for 48 h (Table 1): 1) control, serum-free DMEM; 2) increased amino acids; and 3) L-arginine (2.08 mM). Each of these conditions was also prepared with the addition of the NO synthase inhibitor Nω-monomethyl-L-arginine (L-NAME; 1 mM) to examine whether NO synthesis contributed to the experimental outcomes.

Outcome Measurements

mRNA. Total RNA was isolated from mesangial cell monolayers utilizing Tri Reagent (Molecular Research Center, Cincinnati, OH) and quantified by UV spectrophotometry (Beckman DU 660, Beckman Coulter, Fullerton, CA).

Probes specific for rat sequences published in the GenBank database were obtained by RT-PCR. TGF-β1 cDNA is from nucleotides 818–1315 of sequence X52498. Fibronectin cDNA is from nucleotides 5372–5636 of sequence X15906 (EI1a splice variant associated with TGF-β bioactivity). αs(IV) Collagen is from nucleotides 11–421 of sequence AA924749.

Table 1. Amino acid and glucose concentrations in cell culture media for experimental groups

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Control/High Glucose, mM</th>
<th>Increased Amino Acids/ Combination, mM</th>
<th>Increased L-Arginine, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.6/30.5</td>
<td>5.6/30.5</td>
<td>5.6</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>0</td>
<td>3.101</td>
<td>0</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>0.40</td>
<td>2.48</td>
<td>2.08</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>L-Glycine</td>
<td>0.40</td>
<td>2.23</td>
<td>0.40</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>0.20</td>
<td>0.61</td>
<td>0.20</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0.80</td>
<td>1.41</td>
<td>0.80</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0.80</td>
<td>1.54</td>
<td>0.80</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.80</td>
<td>1.22</td>
<td>0.80</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.20</td>
<td>0.56</td>
<td>0.20</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0.40</td>
<td>0.85</td>
<td>0.40</td>
</tr>
<tr>
<td>L-Proline</td>
<td>0</td>
<td>0.79</td>
<td>0</td>
</tr>
<tr>
<td>L-Serine</td>
<td>0.40</td>
<td>1.04</td>
<td>0.42</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0.80</td>
<td>1.27</td>
<td>0.80</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0.08</td>
<td>0.20</td>
<td>0.08</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>0.42</td>
<td>0.45</td>
<td>0.42</td>
</tr>
<tr>
<td>L-Valine</td>
<td>0.80</td>
<td>1.45</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Amino acid concentrations in control and high-glucose conditions were those found in standard DMEM.
TSP-1 cDNA has been previously described (15). GAPDH is from nucleotides 469–987 of sequences X02231 and X00972. The cDNAs were radiolabeled with [32P]dCTP (Oligolabeling Ready-to-Go kit, Amersham Pharmacia, Piscataway, NJ).

RNA (5 μg) was denaturated, separated by formaldehyde-agarose gel electrophoresis, transferred to charged nylon membranes (Schleicher and Schuell, Keene, NH), and baked at 80°C. Blots were hybridized in Quickhyb (Stratagene, La Jolla, CA) using [32P]-labeled cDNA at 2 × 10⁵ cpm/ml (with rpm being counts/min) for 2 h at 68°C and washed at 60°C with 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate) containing 0.1% SDS. Most of the Northern blots were quantified from digital images obtained with the Cyclone Storage Phosphor System and Optiquant Image Analysis Software (Packard Instruments, Meriden, CT). In several early experiments, Northern blots were exposed to Kodak Biomax MS-1 film (Eastman Kodak, Rochester, NY) for multiple timed exposures. Digital images were obtained with a Duoscan 1200 scanner (AGFA, Wilmington, MA) and analyzed with Kodak 1D Image Analysis Software (Eastman Kodak). Both techniques for quantifying Northern blots yielded comparable results. Quantities of TGF-β1, fibronectin, α1(IV) collagen, and TSP-1 mRNA were expressed as the ratio to GAPDH mRNA.

Proteins. Cell culture media were collected at the end of the experimental periods. Protease inhibitors were added, and media were frozen at −70°C. TGF-β1 protein was quantified by ELISA using a Duoset kit (R&D Systems, Minneapolis, MN), and total TGF-β protein was measured by bioassay (see TGF-β bioactivity). Fibronectin was measured by competition ELISA using rat plasma fibronectin and anti-rat fibronectin antibodies (Chemicon International, Temecula, CA). Standards or unknown samples (100 μl) were incubated with an equal volume of 1:2,000 dilution of rabbit anti-fibronectin antibody in PBS blocking buffer with 3% gelatin (Sigma) for 2 h. The antibody/antigen mixture (100 μl) was transferred to 96-well plates (Rainin Instruments, Oakland, CA), which had been coated overnight with fibronectin (100 ng in 100 μl of 0.5 M NaHCO₃, pH 9.5). The plates were incubated for 2 h, rinsed, and incubated with a 1:20,000 dilution of biotinylated anti-rabbit antibody (Sigma) for 2 h. After the plates were rinsed, they were incubated with a 1:20,000 dilution of biotinylated anti-rabbit antibody (Sigma) for 2 h. After the blots were washed, they were incubated with HRP-conjugated streptavidin-horseradish peroxidase (HRP; Rockland Immunochemicals, Gilbertsville, PA) in PBS. HRP was detected with a substrate reagent (R&D Systems). Total media protein was measured with a Protein Assay Kit (Bio-Rad). Absorbance was measured on a Bio-Tek FL-600 plate reader (Bio-Tek Instruments). Data were reported in digital light units per square millimeter per microgram of cell protein.

NO metabolites. As an index of NO in conditioned media, nitrite and nitrate were measured using a fluorometric NO assay kit (Calbiochem, San Diego, CA). Fluorescence was determined with a Bio-Tek FL-600 fluorescence plate reader (Bio-Tek Instruments) using an excitation wavelength of 365 nm and an emission wavelength of 460 nm. Nitrate/nitrite were expressed as the ratio to total media protein (nmol/μg).

Cell viability. Cell viability was assessed using a In Vitro Toxicology Assay Kit (Sigma) for lactate dehydrogenase (LDH). Cells were grown in 24-well plates and treated as previously described. Conditioned media were collected at the end of experimental periods, and the remaining cell layer was lysed with kit reagent. LDH values obtained from the media and the lysed cell layer were combined for total LDH. Media LDH was expressed as a percentage of total LDH.

Statistics

Data are expressed as means ± SE. One-way ANOVAs were used to evaluate the various outcome measurements in response to experimental conditions. Two-way ANOVAs were used to evaluate differences between the group with increased amino acids and control and TSP-1 peptide groups for total and bioactive TGF-β. Specific tests between conditions were conducted using a priori contrasts. Statistics were computed using SPSS, version 10 (SPSS, Chicago, IL). Probabilities <5% (P < 0.05) were considered statistically significant.

RESULTS

TGF-β1 and Matrix Proteins in Response to Increased Amino Acids, High-Glucose, or the Combination Condition

Increased amino acids enhanced expression of all genes in a manner similar to high glucose (Fig. 1). The
combination condition of increased amino acids and high glucose increased mRNA for TGF-β1, α1(IV) collagen, and TSP-1. A similar trend was noted for fibronectin, but the change from control did not reach statistical significance (P > 0.06). The effects of the combination condition were not significantly different from either elevated levels of glucose or amino acids alone. Mannitol treatment, designed to produce levels of osmolarity similar to experimental conditions, did not increase mRNA for the various proteins (data not shown). TGF-β1 (ELISA), fibronectin, and TSP-1 proteins increased in parallel with the mRNA results (Table 2).

Table 2. Transforming growth factor-β1 and matrix proteins in response to experimental conditions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Increased Amino Acids</th>
<th>High Glucose</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1 (n = 4)</td>
<td>1.28 ± 0.05*</td>
<td>1.17 ± 0.08*</td>
<td>1.14 ± 0.08</td>
</tr>
<tr>
<td>Fibronectin (n = 11)</td>
<td>1.58 ± 0.22*</td>
<td>1.60 ± 0.22†</td>
<td>1.38 ± 0.12†</td>
</tr>
<tr>
<td>TSP-1 (n = 6–8)</td>
<td>1.36 ± 0.08*</td>
<td>1.32 ± 0.08*</td>
<td>1.82 ± 0.29*</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as fold-change from control. n, No. of measurements; TGF-β1, transforming growth factor-1; TSP-1, thrombospondin-1. *P < 0.05, †P < 0.01: increase in experimental groups over control.

TSP-1-Induced Activation of TGF-β by Increased Amino Acids

Both total and bioactive TGF-β (NRK assay) were elevated in the conditioned media of the increased amino acids group (Fig. 2, A and B). Total TGF-β increased 1.88 ± 0.34-fold (P = 0.006), whereas bioactive TGF-β increased 2.68 ± 0.21-fold (P < 0.001). As a result, bioactive TGF-β was proportionally greater in the group that received increased amino acids compared with control [48.9 ± 6.3 vs. 30.5 ± 1.7% (P = 0.021)]. A parallel increase in media fibronectin protein was observed [4.77 ± 0.30 and 2.67 ± 0.20 ng/µg (P < 0.001)] in the increased amino acids and control groups, respectively. The TSP-1 antagonist LSKL peptide decreased the increment in bioactive TGF-β without changing total TGF-β in the increased amino acids group.
group (Fig. 2, A and B). Therefore, the proportion of bioactive TGF-β in the LSKL group was reduced compared with the group that received only increased amino acids [38.7 ± 5.5% (P = 0.014)]. Fibronectin protein was similarly reduced in response to LSKL peptide [2.80 ± 0.20 ng/g (P = 0.001)]. The inert SLLK peptide did not decrease total or bioactive TGF-β (Fig. 2, A and B) or fibronectin protein [3.92 ± 0.29 ng/g (P = 0.115 compared with increased amino acids group)] in conditioned media.

**TGF-β1 and Matrix Proteins in Response to Increased l-Arginine and NO Synthase Inhibition**

Increased l-arginine raised mRNA and protein levels for TGF-β1 (ELISA), fibronectin, and TSP-1, and mRNA for α1(IV) collagen in a manner comparable to the amino acid mixture group (Table 3). Both conditions also resulted in decreased NO metabolites in the conditioned media: 160 ± 18 vs. 125 ± 10 and 101 ± 10 nmol/μg (P = 0.015 and P = 0.025) in control, amino acid mixture, and l-arginine groups, respectively. Addition of the NO synthase inhibitor l-NMMA did not consistently influence TGF-β1 or matrix proteins in either the amino acid mixture or l-arginine condition (Table 3). When the amino acid mixture group also received l-NMMA, mRNA and protein for TGF-β1 and fibronectin and α1(IV) collagen mRNA remained above control levels. For TSP-1, the protein level was greater than control, but the mRNA level was not. None of these values was significantly different from those in the amino acid mixture group that did not receive NO synthase inhibition. When l-NMMA was added to the l-arginine group, TGF-β1 and fibronectin proteins were greater than control, but mRNA levels were not. To the contrary, TSP-1 and α1(IV) collagen mRNA remained greater than control. Although TSP-1 protein was numerically larger than control, it was not statistically significant (P = 0.074). None of these was significantly different from the respective values in the group treated with only l-arginine. In summary, no overall pattern of response to support a role for increased NO production was observed.

**Table 3. Effects of the amino acid mixture or l-arginine alone, with or without nitric oxide synthase inhibition, on TGF-β1 and matrix proteins**

<table>
<thead>
<tr>
<th></th>
<th>Increased Amino Acids</th>
<th>l-Arginine</th>
<th>l-NMMA</th>
<th>l-Arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increased Amino Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β1 mRNA (n = 8–11)</td>
<td>1.47 ± 0.11†</td>
<td>1.58 ± 0.16*</td>
<td>1.66 ± 0.27*</td>
<td>1.08 ± 0.06</td>
</tr>
<tr>
<td>Protein (n = 9–12)</td>
<td>1.41 ± 0.11†</td>
<td>1.31 ± 0.06†</td>
<td>1.27 ± 0.09*</td>
<td>1.21 ± 0.06†</td>
</tr>
<tr>
<td>Fibronectin mRNA (n = 8–11)</td>
<td>1.31 ± 0.13*</td>
<td>1.25 ± 0.13*</td>
<td>1.26 ± 0.07†</td>
<td>1.12 ± 0.11</td>
</tr>
<tr>
<td>Protein (n = 8)</td>
<td>1.58 ± 0.20*</td>
<td>1.56 ± 0.14†</td>
<td>1.28 ± 0.09*</td>
<td>1.36 ± 0.13†</td>
</tr>
<tr>
<td>TSP-1 mRNA (n = 8–11)</td>
<td>1.19 ± 0.06†</td>
<td>1.33 ± 0.08†</td>
<td>1.09 ± 0.10</td>
<td>1.63 ± 0.12*</td>
</tr>
<tr>
<td>Protein (n = 7–8)</td>
<td>1.85 ± 0.29†</td>
<td>1.61 ± 0.14†</td>
<td>2.28 ± 0.58*</td>
<td>2.37 ± 0.35</td>
</tr>
<tr>
<td>α1(IV) Collagen mRNA (n = 6–10)</td>
<td>1.63 ± 0.17*</td>
<td>1.75 ± 0.24*</td>
<td>1.54 ± 0.08*</td>
<td>1.36 ± 0.11*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, †P < 0.01: increase in experimental groups over control.

**Effects of Amino Acids on Mesangial Cell Proliferation and Viability**

The culture of mesangial cells with increased amino acids raised [3H]thymidine incorporation in a manner comparable to that induced by high glucose (Fig. 3). The combination condition increased [3H]thymidine incorporation more than either condition alone. The mixture of amino acids did not adversely affect mesangial cell viability, as determined by LDH release into the media [11 ± 4 vs. 6 ± 1% in control (P = 0.142)]. However, the high-glucose and combination conditions did increase media LDH compared with control [23 ± 2 and 24 ± 5%, respectively (P < 0.001 for both values vs. control)].

**Fig. 3. Increased amino acids, high-glucose, and combination conditions enhance mesangial cell [3H]thymidine incorporation (n = 7).** Values are means ± SE. *P < 0.05 increased amino acids or high-glucose group vs. control. †P < 0.01 combination group vs. control group. ‡P < 0.05 combination group vs. increased amino acids or high-glucose group.
DISCUSSION

The present data demonstrate that amino acids have adverse effects beyond the renal hemodynamic changes long associated with excess protein in the diet. In glomerular mesangial cells, production and activation of TGF-β and expression of matrix proteins (fibronectin, collagen IV, and TSP-1) occurred in response to increased amounts of amino acids designed to replicate those in plasma after protein feeding. The effects of increased amino acids were similar in magnitude to those induced by the high-glucose condition. L-Arginine alone enhanced expression of TGF-β1 and matrix proteins to the same extent as the amino acid mixture and high-glucose conditions. Even though L-arginine is an NO precursor, NO metabolites decreased in the conditioned media of both the L-arginine alone and amino acid mixture groups. In addition, increased levels of amino acids augmented DNA synthesis as much as observed in response to a high-glucose level and, with the combination condition, this index of cellular proliferation was even more pronounced.

High-protein diets exacerbate progressive renal disease, particularly in diabetes (5, 47). Conversely, diets low in protein reduce the progression and, most importantly, decrease the risk of end-stage renal disease and death in diabetic nephropathy (14, 24, 47). Furthermore, when combined with renin-angiotensin system blockade, a low-protein diet has the additive effect of reducing proteinuria (11, 26, 32). These reports and others indicate that dietary protein has effects other than those related to glomerular hypertension or renin-angiotensin system activation, but the mechanisms are still unclear (11, 23, 26, 32). Our data provide new evidence that amino acids have direct cellular effects associated with glomerular injury. However, we cannot exclude involvement of the renin-angiotensin system in the responses of mesangial cells to amino acids. Mesangial cells can produce angiotensin II, especially when stretched or cultured with an increased amount of glucose (4, 38). In addition, in cultured mesangial cells, we recently found that increased amino acids approximately doubled the amount of mRNA for the angiotensin II type 1 receptor and reduced expression of aminopeptidase A, a metalloprotease that degrades angiotensin II (8). These responses could potentiate effects of angiotensin II, including stimulation of TGF-β production and mesangial cell proliferation (2, 18, 29).

Increased mesangial cell DNA synthesis in response to amino acids is another new finding in this study. However, unlike the high-glucose condition, the amino acid mixture did not adversely affect mesangial cell viability, nor did it worsen the reduced cell viability associated with high glucose. In addition to mesangial matrix expansion, cell growth is a common feature of diabetic nephropathy (45). The augmented DNA synthesis in response to the combination condition suggests that, when high circulating levels of glucose and amino acids coexist, a common occurrence in diabetes, mesangial cell proliferation could be exacerbated in an additive fashion. In a recent preliminary report, similar findings were observed for the single amino acid glutamine, alone or in combination with a high-glucose level (35). In our experimental system, glutamine was not further increased by the addition of mixed amino acids because it is not present in the standard solution for hyperalimentation (Travasol). This solution was chosen to produce an in vitro condition similar to clinical hyperaminoacidemia. Nevertheless, even without further increasing glutamine, increases in other amino acids augmented mesangial cell DNA synthesis. These data suggest that common features among amino acids could lead to this consequence.

TGF-β is secreted in an inactive form due to a non-covalent association with the latency-associated peptide. Although the mechanism of activation is not fully understood, TSP-1 can interact with the latency-associated peptide to liberate active TGF-β (20, 30, 33). Either short- or long-term exposure of mesangial cells to a high-glucose level increases TGF-β bioactivity by such a TSP-1-dependent mechanism (28, 46). In the present study, TSP-1 induced by amino acids also activated TGF-β and enhanced production of the matrix protein fibronectin. Of particular interest, amino acid-induced TGF-β production and activation and matrix protein (fibronectin, collagen IV, and TSP-1) expression were nearly identical to responses produced by the high-glucose condition. However, the combination of elevated levels of amino acids and glucose did not further enhance expression of TGF-β1 or these matrix proteins. Taken together, the available data suggest that the mechanism(s) leading to amino acid- or glucose-induced responses may be redundant or shared.

High-glucose concentrations have previously been reported to decrease NO and promote TSP-1-dependent TGF-β bioactivity in mesangial cells (40, 44). Similarly, we found that NO metabolites in the conditioned media decreased when mesangial cells were cultured with either the amino acid mixture or L-arginine. This may seem paradoxical because L-arginine is the precursor for NO. In addition, production of TGF-β1 and matrix proteins increased as much in response to L-arginine as with the amino acid mixture. No clear directional effect of L-NMMA on these outcomes was observed, further supporting the contention that the mechanism(s) in this system is not likely to be dependent on increased NO production. The increase in TGF-β itself could conceivably reduce production of NO by inhibiting transcription of inducible NO synthase, as has been observed in vascular smooth muscle cells stimulated by interleukin-1β (25). However, in mesangial cells, the contribution of inducible NO synthase to the production of NO is uncertain in the absence of cytokine stimulation. The quenching of NO by reactive oxygen species could also explain the decrease in NO metabolites in the conditioned media (6, 16). Indeed, glutamine has recently been reported to enhance superoxide production by neutrophils (27). Similarly, in recent experiments performed in our laboratory, we have found an increase in hydrogen perox-
Amino acids and oxidative stress, which could be a mechanism for quenching NO and promoting injury via the TGF-β pathway. We have demonstrated that amino acids may directly cause or enhance mesangial cell responses associated with fibrosis and progressive renal disease. Such effects could add to glomerular injury, especially in diabetes, a condition where high levels of circulating amino acids, as well as hyperglycemia, are common metabolic disturbances.

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