GLUT-1 Overexpression

Link Between Hemodynamic and Metabolic Factors in Glomerular Injury?

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Abstract—Mesangial matrix deposition is the hallmark of hypertensive and diabetic glomerulopathy. At similar levels of systemic hypertension, Dahl salt-sensitive but not spontaneously hypertensive rats (SHR) develop glomerular hypertension, which is accompanied by upregulation of transforming growth factor β₁ (TGF-β₁), mesangial matrix expansion, and sclerosis. GLUT-1 is ubiquitously expressed and is the predominant glucose transporter in mesangial cells. In mesangial cells in vitro, GLUT-1 overexpression increases basal glucose transport, resulting in excess fibronectin and collagen production. TGF-β₁ has been shown to upregulate GLUT-1 expression. We demonstrated that in hypertensive Dahl salt-sensitive (S) rats fed 4% NaCl (systolic blood pressure [SBP]: 236 ± 9 mm Hg), but not in similarly hypertensive SHR (SBP: 230 ± 10 mm Hg) or their normotensive counterparts (Dahl S fed 0.5% NaCl, SBP: 145 ± 5 mm Hg; and Wistar-Kyoto, SBP: 137 ± 3 mm Hg), there was an 80% upregulation of glomerular GLUT-1 protein expression (P ≤ 0.03). This was accompanied by a 2.7-fold upregulation of TGF-β₁ protein expression in glomeruli of DSH compared with DSN rats (P ≤ 0.02). TGF-β₁ expression was not upregulated and did not differ in the glomeruli of Wistar-Kyoto and SHR rats. As an in vitro surrogate of the in vivo hemodynamic stress imposed by glomerular hypertension, we used mechanical stretching of human and rat mesangial cells. We found that after 33 hours of stretching, mesangial cells overexpressed GLUT-1 (40%) and showed an increase in basal glucose transport of similar magnitude (both P ≤ 0.01), which could be blocked with an anti TGF-β₁-neutralizing antibody. These studies suggest a novel link between hemodynamic and metabolic factors that may cooperate in inducing progressive glomerular injury in conditions characterized by glomerular hypertension. (Hypertension. 2003;42:19-24.)

Key Words: hypertension, experimental ■ mesangium ■ stress ■ transforming growth factors

In hypertensive glomerular injury, mesangial expansion with extracellular matrix accumulation is one of the hallmarks of glomerulosclerosis.1,2 Similarly, mesangial expansion is a central feature of diabetic glomerulopathy, in which both hemodynamic and metabolic perturbations play a crucial role in the pathogenesis of this disease.3 Mesangial cells express primarily 2 types of glucose transporters: the facilitative brain type glucose transporter GLUT-1, the predominant isoform, and the sodium-coupled glucose transporter.4 GLUT-1 is ubiquitously expressed, and its cellular distribution resides mainly in the cellular plasma membrane, where it plays a major role in the basal rate of glucose transport into the cell.5 This is particularly relevant for glucose metabolism of cells such as mesangial cells in which glucose uptake is relatively insulin-independent.4 As with all the other members of the facilitative glucose transport family, GLUT-1 is a high-affinity, low-capacity glucose transporter and is at or near saturation at physiological glucose levels. Therefore, changes in GLUT-1 expression, translocation, or intrinsic activity are necessary for cells to significantly increase their basal glucose uptake.6

Overexpression of GLUT-1 in rat mesangial cells cultured in normal glucose conditions results in increased basal glucose transport accompanied by induction of fibronectin and of collagen expression, mimicking the effect of high glucose.7 Conversely, overexpression of an antisense GLUT-1 mRNA in mesangial cells leads to reduction of glucose uptake and prevention of glucose-induced fibronectin.8

In hypertensive as well as in diabetic glomerulopathy, similar signaling pathways and cytokines are upregulated, notably transforming growth factor β₁ (TGF-β₁).9,10 TGF-β₁ has been shown to upregulate GLUT-1 mRNA and protein expression and basal glucose transport in mesangial cells in culture.11

Received December 9, 2003; first decision December 26, 2002; revision accepted April 29, 2003.

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Hypertension is available at http://www.hypertensionaha.org DOI: 10.1161/01.HYP.0000075949.19968.EF
Animal models and humans with combined hypertension and diabetes show the most severe glomerular lesions, therefore, reduction of glomerular pressure, with agents that interrupt the renin-angiotensin system, downregulates TGF-β, and specifically delays the progression toward renal failure. Whether intraglomerular pressure and increased glucose uptake operate independently or work in synergy to produce glomerular damage is unknown. We hypothesized that the injurious effect of the hemodynamic stress of glomerular hypertension could be mediated, at least in part, by upregulation of GLUT-1 expression resulting in a pathologically increased glucose uptake.

We tested this hypothesis by comparing GLUT-1 expression in hypertensive Dahl salt-sensitive rats and spontaneously hypertensive rats (SHR) matched by age and severity of hypertension. We have previously shown that in Dahl salt-sensitive rats but not in SHR, systemic hypertension is accompanied by glomerular hypertension and injury. In addition, in studies in vitro, we investigated whether, in mesangial cells, mechanical stretch upregulates GLUT-1, leading to an increased transport of glucose.

Methods

Chemicals and Reagents
All chemicals were purchased from Sigma unless otherwise specified. Fetal calf serum was purchased from Gibco BRL and [3H] 2-deoxyglucose from Amershams. Animals were provided by Harlan SD.

Animals
Male Dahl salt-sensitive (DS) rats, SHR, and Wistar-Kyoto (WKY) rats were studied between 11 and 12 weeks of age. DS rats were fed from the age of 4 weeks a low salt (0.5%) diet, DSN normotensive control for the SHR. Male Dahl salt-sensitive (DS) rats, SHR, and Wistar-Kyoto (WKY) rats were studied between 11 and 12 weeks of age. DS rats were fed from the age of 4 weeks a low salt (0.5%) diet, DSN normotensive control, or a high salt (4%) diet, DSH hypertensive animals.

DSH were used as a model of systemic and intraglomerular hypertension; SHR served as a model of systemic hypertension without intraglomerular hypertension. The WKY was used as the normotensive control for the SHR.

All rats were maintained in a 24 °C environment, with a 12 hour light/dark cycle, and fed ad libitum with water and standard chow diet.

Before the animals were killed, systolic blood pressure was measured in unanesthetized rats with a tail noninvasive blood pressure determination system, and proteinuria was quantified in 24-hour urine collections from rats individually kept in metabolic cages, using a BioRad protein assay kit.

Immediately after death, frozen kidney tissue for immunohistochemistry analysis and isolated glomeruli were obtained. Glomeruli were obtained by sequential sieving from kidneys of each individual animal. After sieving, the glomeruli were collected in a sterile universal container and washed 3 times with cold PBS to preclude tubuli contamination.

Cell Culture
Human mesangial cells (HMC) were obtained from glomeruli isolated from tumor-free portions of tumor nephrectomy specimens or from donor kidneys unsuitable for transplantation by serial sieving of renal cortex followed by collagenase digestion, as previously reported. Rat mesangial cells (RMC) were obtained from a cloned cell line (16KCC2) derived from the outgrowth of rat glomeruli and previously characterized. Cells were cultured in RPMI 1640 medium, 7 mmol/L glucose, supplemented with insulin-transferrin-selenium (ITS), 20% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO2 incubator at 37°C and the experiment performed in cells grown on collagen type I.

Subconfluent mesangial cells were serum-starved (0.5% serum) for 12 hours and subsequently exposed to mechanical stretch (60 cycles/min) at 10% average cell elongation for 12, 24, 30, and 33 hours (Flexercell 3000 Strain Unit). Control unstretched cells were studied in parallel. In HMC, experiments were conducted in the presence of a neutralizing anti–TGF-β, antibody (200 ng/mL) (R&D System) or control nonimmune IgG.

Immunohistochemistry
Kidney tissue was frozen in isopentane, cooled in liquid nitrogen, and stored at −80°C. GLUT-1 immunoperoxidase staining was carried out on 4-μm frozen tissue sections fixed in acetone at 4°C for 10 minutes. Tissue sections were washed in 0.2% Tween Tris buffer saline (TTBS), pH 7.4, followed by peroxidase blocking step with 0.03% hydrogen peroxide for 10 minutes. The sections were blocked for 30 minutes with 10% goat serum in TTBS, and this was followed by 1-hour incubation at room temperature with primary rabbit polyclonal anti GLUT-1 antibody (1:600 dilution)(Alpha Diagnostic). Sections were subsequently incubated with a peroxidase-labeled polymer conjugated to a goat anti-rabbit IgG (Dako) for 30 minutes. After intermediate washes with TTBS, visualization of GLUT-1 was obtained with 3, 3′-diaminobenzidine (brown staining). The sections were counterstained with hematoxylin to visualize nuclei, and finally were mounted in mounting medium. Negative controls were prepared by either omitting the anti–GLUT-1 antibody or by the addition of specific GLUT-1–blocking peptide (Alpha Diagnostic).

Fifteen randomly selected glomeruli were analyzed for each animal by a masked observer; the calculated average GLUT-1 staining was then used for statistical analysis. The proportional area occupied by immunoreactive GLUT-1 was calculated by using a computer-assisted image analysis system KS-300 (Zeiss) connected to a BX60 microscope (Olympus) and a KY-F55B (JVC) color videocamera. Glomerular boundaries were defined by external perimeter of the capillary loops as previously described. Determinations were made at the same light microscope intensity.

GLUT-1 and TGF-β Protein Expression Determination
Total protein from both rat glomeruli and mesangial cells were extracted with the use of a lysis buffer containing 50 mmol/L Tris-HCl, pH 7.6, with 1% Triton, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonyl-fluoride (PMFS), and 100 mmol/L sodium chloride. Lysates were then sonicated for 45 seconds at 4°C. Each lane was loaded with 60 μg total cell lysate.

GLUT-1 and TGF-β protein expression was studied by Western immunoblotting with specific GLUT-1 antisera (1:2500) (Alpha Diagnostic) and TGF-β, antisera (1:6000) (Santa Cruz Biotechnology, Inc.). Both antisera specifically bind, respectively, to GLUT-1 and TGF-β, from rat tissues, and they have been widely validated in previous work.

Equal loading was confirmed by Ponceau staining of the proteins transferred onto the nitrocellulose membrane. After enhanced chemiluminescence, band intensity was quantified by densitometry.

Glucose Transport
Glucose transport assay in mesangial cells in vitro was performed as previously described, with few modifications. In brief, basal transport was started by the addition of 100 μmol/L 2-deoxyglucose (2-DG) with ~0.4 μCi/well of [3H] 2-DG in glucose-free minimal essential media. Transport was performed at 37°C for 5 minutes and stopped with the addition of 1 mL phlorizin solution in PBS (82 mg/mL) (preliminary experiments showed a linear 2-DG uptake up to 15 minutes, data not shown). 2-DG incorporation into cells was measured with a β-counter. 2-DG uptake was calculated after subtraction of 2-DG–nonspecific uptake in parallel samples incubated in the presence of 10 μmol/L cytochalasin B. Results were normalized for cell number determined in parallel wells.

Statistical Analysis
Differences among groups were analyzed by repeated-measures ANOVA. Post hoc pairwise comparisons were performed by using least significant differences and Dunnett test methods. A Student t
test was used to compare DSH or SHR with their respective controls, specifically for immunohistochemistry and immunoblotting determinations. Statistical significance was accepted at a level of \( P < 0.05 \). Results are reported as mean±SEM unless otherwise stated.

**Results**

### In Vivo Studies

#### Rat Characteristics

Body weight was similar in all groups of rats studied (DSH, 349.3±63 g; DSN, 332±53 g; SHR, 285±33 g; WKY, 293±53 g, n=4). Systolic blood pressure was similar between DSH (236±9 mm Hg, n=4) and SHR (230±10 mm Hg, n=4) but significantly elevated when compared with their respective normotensive controls DSN (145±5 mm Hg, n=4) and WKY (137±3 mm Hg, n=4) rats. Twenty-four-hour urine protein excretion in the DSH (147±23 mg/24 h, n=4) was 5-fold higher than in the normotensive DSN rats (36.6±11 mg/24 h, n=4). Proteinuria in the SHR (8.5±3 mg/24 h, n=6) was lower than the DSH; normotensive WKY did not show significant proteinuria.

**GLUT-1 Is Upregulated in Glomeruli of DSH Rat by Immunohistochemistry in Renal Cortex and by Immunoblotting in Isolated Glomeruli**

By immunohistochemistry renal cortex analysis, the percent change in staining for GLUT-1 per glomerular area was significantly higher in DSH than DSN rats (182±19.7\% versus 100±22.1\%, respectively, \( P = 0.03 \)), whereas no difference was found between SHR and WKY rats (109.6±5.8\% versus 100±6.4\%, respectively). The staining pattern in the glomeruli of DSH rats suggested GLUT-1 upregulation in different glomerular cell types, including the mesangium. GLUT-1 immunostaining was present in the tubules of all animals. No staining was observed when the first antibody was omitted or blocking peptide added. Figure 1 shows representative glomeruli from the 4 animal groups.

Consistent with this, glomerular GLUT-1 protein expression was 80\% greater in the DSH rats than in DSN rats (\( P = 0.004 \)) as assessed by Western blotting, whereas no difference was seen between SHR and WKY (Figure 2).

**TGF-β1 Is Upregulated in Isolated Glomeruli of DSH Rat**

Immunoblotting with specific anti-TGF-β1 antiserum in total cell lysate of isolated glomeruli from the 4 animal groups showed that TGF-β1 expression was 2.7-fold higher in DSH rats compared with DSN rats. By contrast, expression of TGF-β1 was virtually undetectable in both WKY and SHR rats, and there were no differences between the 2 groups (Figure 3).

### In Vitro Studies

#### Mechanical Stretch Upregulates GLUT-1 Protein Level in Human Mesangial Cells

We studied the effect of mechanical stretch on GLUT-1 protein expression in HMC stretched for 12, 24, and 33 hours. Exposure to mechanical stretch for 33 hours significantly increased GLUT-1 protein level by 40\% over control animals (\( P = 0.01 \)) (Figure 4a), whereas no significant changes were observed at earlier time points (arbitrary units percentage...
Mechanical Stretch Upregulates Glucose Transport in Human and Rat Mesangial Cells

To investigate whether GLUT-1 upregulation was paralleled by an increase in cellular glucose uptake, 2-DOG basal glucose transport assay was performed after 33 hours of stretch when GLUT-1 upregulation was observed. There was a significant 60% increase \( (P<0.002) \) in 2-DOG uptake in HMC exposed to stretch as compared with control nonstretched cells (Figure 4b). A similar significant increase, although less in magnitude, was observed in stretched RMC (arbitrary unit % change over control nonstretched cells=100%, at 30 to 33 hours, 122.5±4.3%, \( P=0.005 \)), indicating that this phenomenon is common in both species.

TGF-β1 Mediates Stretch-Induced GLUT-1 and Basal Glucose Transport Upregulation

To elucidate the mechanism of stretch-induced GLUT-1 and basal glucose transport upregulation in HMC, we studied GLUT-1 expression and basal 2-DOG glucose uptake in mesangial cells stretched either in the presence or absence of a specific TGF-β1–neutralizing antibody. TGF-β1 blockade significantly blunted by \( \approx 80\% \) both the stretch-induced GLUT-1 protein expression \( (P=0.04) \) and basal glucose transport \( (P=0.006) \), observed at 33 hours. No change was detected when the control IgG was added (Figures 4a and 4b). Thus, stretch-induced GLUT-1 overexpression and basal glucose transport appear largely mediated by TGF-β1.

Discussion

In this study, we have demonstrated that in hypertensive DSH rats but not in similarly hypertensive SHR there is 80% increase in glomerular GLUT-1 both by immunohistochemistry and by Western blot; GLUT-1 levels in normotensive WKY and DSN were similar to those in SHR.

These findings suggested that GLUT-1 upregulation might be a consequence of the hemodynamic stress imposed by glomerular hypertension. It has been previously shown that

Figure 3. TGF-β1 protein level in glomeruli isolated from WKY, SHR, DSN, and DSH rats. Upper panel shows representative Western immunoblotting; lower panel shows densitometry analysis for TGF-β1 expressed as percentage change over control (WKY and DSN, respectively). \( \ast P=0.02, \) DSH vs DSN (n=4 animals per group).

Figure 4. GLUT-1 protein expression (a) and basal glucose transport (b) in 33 hours stretched (s) and nonstretched (ns) HMC in the presence or absence of anti–TGF-β1–neutralizing antibody or control IgG. Data are expressed as percentage change over nonstretched cells (experiments were n=5 performed in duplicate for glucose transport, and n=5 for GLUT-1 protein). \( \ast P<0.05 \) for stretch control IgG vs other conditions; \( \dagger P<0.01 \) for stretch control IgG vs other conditions. Representative Western immunoblotting for GLUT-1 protein is shown in (a).
transmission of the elevated systemic blood pressure to the glomerular circulation in the DSH rat results in glomerular hypertension, mesangial matrix expansion, and development of glomerulosclerosis. In contrast, in the SHR, increased preglomerular resistances prevent glomerular capillary pressure from rising in response to systemic hypertension, and glomerular damage is modest and delayed. When the preglomerular resistances are reduced such as in uninephrectomized or in the diabetic SHR, the development of glomerular capillary hypertension is associated with the development of accelerated proteinuria and an increased mesangial expansion and glomerulosclerosis.

Previous studies have reported an increased expression of TGF- in the glomeruli of hypertensive Dahl salt-sensitive rats treated with a high salt diet. In contrast, no differences have been observed in TGF- expression in SHR rats between 7 and 16 weeks of age when compared with normotensive WKY rats.

In this study, we confirmed that TGF- is upregulated in isolated glomeruli of DSH rats, suggesting that the upregulation of TGF- may account for the increase of GLUT-1 expression that we have observed.

This interpretation of the in vitro findings is consistent with our results in vitro, which show that mesangial cells, an important target for mechanically induced glomerular injury, when subjected to mechanical stretch, upregulate GLUT-1 protein expression as well as basal glucose transport through a TGF- dependent mechanism.

These data are in accord with previous observations both in vivo and in vitro. In DSH rats, reduction of intraglomerular pressure with an angiotensin II antagonist prevents glomerular TGF- upregulation. Further systemic treatment with anti-TGF- antibodies in hypertensive Dahl rats reduces blood pressure as well as proteinuria and the severity of glomerulosclerosis.

In this context, the finding that diabetic animals with incipient diabetic nephropathy have increased urinary excretion of TGF-1, which is associated with greater abundance of renal cortical GLUT-1 protein, is notable.

In cultured mesangial cells mechanical stretch upregulates TGF- levels and activity. Furthermore, addition of TGF- to mesangial cells in culture increases basal glucose transport by stimulating GLUT-1 expression both at the mRNA and protein levels.

In mesangial cells, high glucose also upregulates GLUT-1, an event believed to represent one of the mechanisms of glomerular injury in diabetes. This glucose-induced GLUT-1 upregulation is, at least in part, mediated by TGF-1. On the other hand, GLUT-1 overexpression in mesangial cells in normal glucose medium leads to excess extracellular matrix production.

This is the first demonstration that mechanical stretch in normal ambient glucose concentration leads to increased glucose transport through TGF- dependent upregulation of GLUT-1. Collectively, our findings suggest a possible mechanism of interaction between mechanical forces and glucose-mediated pathways in the pathogenesis of glomerular injury.

Hypertension is a major risk factor in the progression of renal disease in general and diabetic nephropathy in particular. It is known that agents that interrupt the renin-angiotensin system are particularly renoprotective. These agents reduce systemic as well as glomerular hypertension and, by downregulating TGF-1, are likely to interrupt the TGF-GLUT-1 axis. Our present work describes a novel pathophysiological mechanism that may be operative in renal disease accompanied by glomerular hypertension.

Perspectives
The studies reported herein, by linking mechanical stretch with glucose transport/metabolism, suggest a novel pathophysiological mechanism of injury in hypertensive glomerular diseases. Clinically, these studies may lead to the development of new therapeutic strategies in hypertensive glomerulopathies.

Acknowledgments
This work was supported by Diabetes UK Grants RD/98/0001608 (R.D. Lawrence Fellowship to Dr. Gruden), 2308/0001862 (equipment grant to Dr. Gruden), RD/98/0001759 (project grant for Miss Burt), and RD/98/0001861 (project grant for Dr. Rodriguez); and a grant from Department of Veterans Affairs to Dr. Raij. We thank Mr. Brian Johnston and Mr. Mark Blades for technical assistance.

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Hypertension. 2003;42:19-24; originally published online May 27, 2003;
doi: 10.1161/01.HYP.0000075949.19968.EF
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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