Transforming Growth Factor-β, 20-HETE Interaction, and Glomerular Injury in Dahl Salt-Sensitive Rats

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Abstract—This study examined the role of transforming growth factor-β (TGF-β) in altering the glomerular permeability to albumin (Palb) during hypertension development in Dahl salt-sensitive (Dahl S) rats and whether TGF-β acts by inhibiting the glomerular production of 20-HETE. The results indicate that the renal expression of TGF-β doubles in Dahl S rats fed a high-salt diet for 7 days, and this is associated with a marked rise in Palb from 0.19±0.04 to 0.75±0.01 and changes in the ultrastructure of the glomerular filtration barrier. Chronic treatment of Dahl S rats with a TGF-β neutralizing antibody prevented the increase in Palb and preserved the structure of glomerular capillaries. It had no effect on the rise in blood pressure produced by the high-salt diet. In other studies, preincubation of glomeruli isolated from Sprague Dawley rats with TGF-β1 (10 ng/mL) for 15 minutes increased Palb from 0.01±0.01 to 0.60±0.02. This was associated with inhibition of the glomerular production of 20-HETE from 221±11 to 3.4±0.5 µg per 30 minutes per milligram of protein. Pretreatment of Sprague Dawley glomeruli with a stable analog of 20-HETE, 20-hydroxyeicosatetraenoic acid, reduced baseline Palb and opposed the effects of TGF-β to increase Palb. These studies indicate that upregulation of the glomerular formation of TGF-β may contribute to the development of proteinuria and glomerular injury early in hypertension development in Dahl S rats by increasing Palb through inhibition of the glomerular production of 20-HETE. (Hypertension. 2005;45:1-6.)

Key Words: transforming growth factors ■ kidney ■ hypertension, renal

Dahl salt-sensitive (Dahl S) rats exhibit many traits associated with salt-sensitive hypertension in humans.1,2 They are salt sensitive,3,4 insulin resistant,5 and hyperlipidemic,6,7 and they rapidly develop proteinuria and glomerulosclerosis when challenged with a high-salt (HS) diet.7-11 The glomerular lesions that develop resemble those seen in patients with hypertension- and diabetes-induced nephropathy.12-14 However, the factors that contribute to the pathogenesis of hypertension-induced glomerulosclerosis remain to be determined.

Recent studies have indicated that the renal expression of transforming growth factor-β (TGF-β) is elevated in Dahl S rats15 and that upregulation of the glomerular formation of TGF-β may contribute to the development of proteinuria and glomerular injury early in hypertension development in Dahl S rats by increasing Palb during hypertension development in Dahl S rats and whether TGF-β may act in part by inhibiting the glomerular production of 20-HETE.

Materials and Methods

General

Experiments were performed on 7-week-old Sprague Dawley (Taconic Labs) rats fed a normal-salt diet containing 1% NaCl (5010; Purina) and Dahl salt-sensitive/John Rapp rats obtained from our colony maintained at the Medical College of Wisconsin. Rats were fed a purified diet (AIN76) purchased from Dytes, Inc. that contained either 0.4% (low-salt [LS]) or 8.0% NaCl (HS). To assess the role of TGF-β in altering the glomerular Psl, during hypertension development in Dahl S rats and whether TGF-β may act in part by inhibiting the glomerular production of 20-HETE.
ment, a group of the Dahl S rats fed an HS diet were treated with an intraperitoneal injection of a murine anti–TGF-β monoclonal Ab (0.5 mg/kg; 1D11; Genzyme Corp) or a control murine monoclonal Ab (13C4; antiverotoxin) every other day. At the end of the treatment period, rats were placed overnight in metabolic cages for measurement of protein and albumin excretion. They were then anesthetized with halothane, and the kidneys were collected for measurement of the expression of TGF-β protein levels by Western blot and for glomerular isolation for measurement of Palb and production of 20-HETE. Catheters connected to radiotelemetry transmitters (Data Science Inc.) were implanted into the femoral artery of 10 additional control and 10 1D11-treated Dahl S rats to determine the effects of anti–TGF-β therapy on the development of hypertension. Mean arterial pressure (MAP) was measured for 3 hours per day, between 9 AM and 12 PM, during a control period when rats were fed an LS diet and after they were fed an HS diet for 7 days. All protocols were approved by the institutional animal welfare committee of the Medical College of Wisconsin.

**Measurement of P_{ab}**

Glomeruli were isolated using the sieving method as described previously in a medium containing 5 g/dL of BSA. In each experimental condition, P_{ab} was determined from the change in glomerular volume (ΔV) after exchange of the bath with medium containing 1 g/dL albumin. P_{ab} was calculated as 

\[ P_{ab} = \frac{\Delta V_{experimental}}{\Delta V_{control}} \]

where glomeruli from Sprague Dawley rats fed a normal-salt diet were used to provide the control value for each experiment. To verify that lack of ΔVs in Dahl S rats were related to changes in P_{ab}, rather than to changes in mechanical properties of glomeruli, additional studies were performed in which the glomeruli were exposed to a 5% solution of high molecular weight dextran. A change in the size of Dahl S glomeruli under these conditions indicates that the lack of response to 1% albumin was attributable to an increase in P_{ab}.

In other experiments, we examined the interaction of TGF-β and 20-HETE on P_{ab} in glomeruli isolated from Sprague Dawley rats and Dahl S rats fed either an LS diet or an HS diet for 4 days. Glomeruli were preincubated with vehicle or TGF-β1 (10 ng/mL) for 15 minutes at 37°C, and changes in P_{ab} were determined. Glomeruli were also pretreated with a stable 20-HETE agonist, 20-hydroxyeicosose-5(Z),14(Z)-diene acid (WIT003: 1 μmol/L; Taisho Pharmaceutical), for 15 minutes at 37°C, and the P_{ab} response to TGF-β1 (10 ng/mL) was redetermined. A minimum of 5-8 glomeruli from each rat were studied, and these experiments were performed using ≥5 rats per treatment group.

**Electron Microscopy**

Kidneys from Dahl S rats fed an LS diet and Dahl S rats fed an HS diet for 1 week and treated with 1D11 or vehicle were collected and fixed in 4% glutaraldehyde solution. Thin epon sections were prepared, stained with uranyl acetate and lead citrate, and examined at 16,000× using a transmission electron microscope (Hitachi H600).

**Western Blots**

Homogenates were prepared from the kidneys of control Sprague Dawley rats and Dahl S rats fed an LS or HS diet for 7 days. Aliquots of the homogenates (30 μg protein) were separated on a 12.5% sodium dodecyl sulfate gel, transferred to a nitrocellulose membrane incubated with a primary TGF-β1 Ab (SC:146; Santa Cruz Biotechnology), followed by a secondary Ab (SC:2004; Santa Cruz Biotechnology), and developed using enhanced chemiluminescence as described previously. Membranes were poststained with Coomassie blue to normalize results for potential differences in sample loading.

**Liquid Chromatography/Mass Spectroscopy**

**Measurement of Glomerular 20-HETE Production**

Glomeruli (~20 μg protein) were incubated in a 0.1 mol/L KPO4 buffer containing 1 mmol/L NADPH for 30 minutes at 37°C in the presence and absence of TGF-β1 (10 ng/mL). Incubations were stopped by acidification with formic acid, homogenized, and the homogenate extracted with chloroform:methanol (2:1) after addition of 10 ng of internal standard, 14,15-epoxyeicosa-5(Z)-enoic-methyl sulfonylimide (EEZE). Samples were reconstituted in 50% acetonitrile, cleaned using an online reverse-phase high-performance liquid chromatography (HPLC) trapping column, and then the HETEs and epoxyeicosatrienoic acids (EETs) in the samples were separated using an isocratic step gradient on an 18C-RP-2×250 mm microbore HPLC (150×21 3 μm; BetaBasic18; Thermo.Hypersil-Keystone) using a mobile phase consisting of acetonitrile:water:acetic acid (57:43:0.1) for 20 minutes to resolve the HETEs followed by acetonitrile:water:acetic acid (63:37:0.1) for 15 minutes to resolve the EETs. Samples were ionized using negative ion electrospray and the peaks eluting with a mass/charge ratio (m/z) of 319 (HETEs and EETs) or 323 (internal standard) were isolated and monitored in the selective ion mass spectroscopy (MS) mode using an Agilent LSD ion trap mass spectrometer (Agilent Technologies 1100). The ratio of ion abundances in the peaks of interest (HETEs and EETs; m/z 319) versus that corresponding to the closely eluting internal standard (EEZE; m/z 323) was determined and compared with a standard curve generated over a range from 0.1 to 2 ng of 20-HETE and EETs with each batch of samples.

**Statistics**

Mean values ±1 SE are presented. Significance of differences between mean values was determined using an ANOVA followed by the Student-Newman–Keuls post hoc test. A P<0.05 was considered significant.

**Results**

**Effects of HS Diet on the Renal Expression of TGF-β1**

The results of these experiments are presented in Figure 1. The expression of TGF-β1 in the kidney more than doubled...
in Dahl S rats fed an HS diet for 1 week compared with the levels seen in Dahl S rats fed an LS diet.

Effects of HS Diet on $P_{\text{alb}}$
A comparison of $P_{\text{alb}}$ in Sprague Dawley and Dahl S rats fed an LS and HS diet at various times for up to a week are presented in Figure 2. Baseline $P_{\text{alb}}$ was significantly higher in Dahl S rats maintained on an LS diet than in control Sprague Dawley rats. $P_{\text{alb}}$ increased in Dahl S rats fed an HS diet after only 4 days, and it reached a peak after 7 days. The increase in $P_{\text{alb}}$ in Dahl S rats fed an HS for 1 week was associated with a significant rise in blood pressure from 121±2 to 136±3 mm Hg (n=10) and a marked increase in the excretion of protein from 47±8 mg per day to 217±31 mg per day (n=14). Similarly, albumin excretion rose from 27±9 mg per day to 129±26 mg per day, respectively, after Dahl S rats were fed an HS diet for 7 days.

Role of TGF-$\beta$ in Altering $P_{\text{alb}}$ in Dahl S Rats
A comparison of the effects of exogenous administration of TGF-$\beta 1$ (10 ng/mL) on $P_{\text{alb}}$ in glomeruli isolated from Sprague Dawley and Dahl S rats is also summarized in Figure 2. TGF-$\beta 1$ increased $P_{\text{alb}}$ from 0.01±0.01 to 0.56±0.02 in glomeruli isolated from Sprague Dawley rats and from 0.19±0.01 to 0.75±0.01 in glomeruli isolated from Dahl S rats fed an LS diet. TGF-$\beta 1$ also increased $P_{\text{alb}}$ in Dahl S rats fed an HS diet for 4 days, but it had no effect on $P_{\text{alb}}$ in Dahl S rats fed an HS diet for 7 days because the baseline $P_{\text{alb}}$ in these rats was already near maximal.

Chronic treatment of Dahl S rats fed an HS diet with a TGF-$\beta$ neutralizing Ab prevented the increase in baseline $P_{\text{alb}}$. Administration of TGF-$\beta 1$ to these glomeruli still increased $P_{\text{alb}}$, similar to that seen in glomeruli isolated from control Sprague Dawley rats and Dahl S rats fed an LS diet. TGF-$\beta$

Figure 2. Effect of an HS diet and the role of TGF-$\beta$ on $P_{\text{alb}}$ in glomeruli isolated from Sprague Dawley rats and Dahl S rats fed an LS and HS diet for 7 days or in Dahl S rats fed an HS diet that were treated with a TGF-$\beta$ Ab (1D11-7). The TGF-$\beta$ Ab (1D11) is against all 3 isoforms of TGF-$\beta$. Glomeruli were preincubated vehicle or 10 ng/mL of TGF-$\beta 1$ for 15 minutes at 37°C and $P_{\text{alb}}$ was measured. Numbers in parentheses indicate the number of glomeruli and number of rats studied per group. *Significant difference vs the values seen in Dahl S rats fed an LS diet; †significant difference from the corresponding control value; SD, Sprague Dawley; HS-4, HS for 4 days; HS-7, HS for 7 days.

Figure 3. Representative electron micrographs of the ultrastructure of the glomerular filtration barrier in Dahl S rats fed an LS or HS diet for 1 week or in Dahl S rats fed an HS diet that were treated with a TGF-$\beta$ Ab. A presents results from a Dahl S rats fed an LS diet. B presents results from Dahl S rats fed an HS diet. C presents results from Dahl S rats fed an HS diet treated with TGF-$\beta$ Ab.

Ab therapy had no effect on the rise in blood pressure. Blood pressure rose from 123±4 to 136±3 mm Hg (n=10) in Dahl S rats fed an HS diet that were treated with 1D11 for 7 days.

Electron Microscopy
Representative electron micrographs of the ultrastructure of glomerular capillaries in Dahl S rats fed an LS or HS diet, and in those treated with the TGF-$\beta$ Ab for 1 week, are presented in Figure 3. The Dahl S rats fed an LS diet exhibited a normal appearance of the glomerular ultrafiltration barrier (Figure 3A). In Dahl S rats fed an HS diet for 7 days (Figure 3B), there was a retraction and fusion of the foot processes of podocytes and exposure of portions of the basement membrane. There was also swelling of the endothelial cells lining the glomerular capillaries, which changed their shape from a flattened to a more cuboidal endothelium. These changes in the ultrastructure of glomerular filtration barrier in Dahl S rats fed an HS diet were prevented by administration of the TGF-$\beta$ Ab (Figure 3C).

Effect of TGF-$\beta$ on Glomerular Production of 20-HETE
The effects of TGF-$\beta$ on the production and metabolism of arachidonic acid (AA) by isolated glomeruli are presented in Figure 4. Glomeruli incubated with AA produced a number of large peaks with an m/z of 319 that coelute with 20-HETE, 15-HETE, 12-HETE, 5-HETE and 14,15-EET, 11,12-EET, 8,9-EET, and 5,6-EET standards (Figure 4A). We further
verified that the largest peak that elutes at 16 minutes after fragmentation produces an MS/MS spectrum with prominent secondary ions at m/z of 301, 273, 257, and 245, identical to that seen with a 20-HETE standard. Pretreatment of glomeruli with TGF-β1 selectively reduced formation of 20-HETE by 97% (Figure 4B) without affecting the formation of 15-HETE, 12-HETE, or 5-HETE, or EETs (Figure 4A).

**Effects of a 20-HETE Agonist on P alb**

The effect of addition of a 20-HETE agonist on P alb is summarized in Figure 5. Pretreatment of glomeruli with a 20-HETE agonist reduced baseline P alb and greatly attenuated the increase in P alb of glomeruli isolated from Dahl S rats fed an HS diet for 4 days. For example, TGF-β1 increased P alb from 0.58±0.04 (n=25 glomeruli; 5 rats) to 0.87±0.02 (n=25; 5) in glomeruli isolated from Dahl S rats fed an HS diet for 4 days. After pretreatment of glomeruli with the 20-HETE agonist, TGF-β1 P alb only increased from 0.25±0.01 (n=25; 5) to 0.40±0.01 (n=25; 5).

**Discussion**

The present study examined the role of TGF-β in altering P alb in Dahl S rats fed an HS diet. TGF-β stimulated P alb by a direct signaling event distinct from the longer-term responses to TGF-β that alter gene expression and time course of the changes in P alb correspond with the rise in MAP, which increased by 15 mm Hg over 7 days in the present study. The change in P alb in Dahl S rats fed an HS diet was associated with parallel increases in proteinuria and albuminuria. We found that there was retraction and fusion of foot processes of podocytes, leading to denudation of portions of the glomerular basement membrane. There also was swelling of the endothelial cells lining the glomerular capillaries in Dahl S rats fed an HS diet for 7 days. These changes are consistent with the increase in P alb seen in these rats.

Additional experiments were designed to explore the role of TGF-β in mediating the increase in P alb in the glomeruli of Dahl S rats fed an HS diet. Consistent with previous findings,30 expression of TGF-β protein was elevated very early during hypertension development in the kidneys of Dahl S rats fed an HS diet for 7 days. Chronic treatment of these rats with a TGF-β Ab, which neutralizes all 3 isoforms of TGF-β, prevented the increase in P alb and the fusion of the foot processes of the podocytes along the basement membrane. These findings suggest that an elevation in the production of TGF-β in the glomerulus plays an important role in increasing P alb during hypertension development in Dahl S rats. The signal triggering the increase in the glomerular production of TGF-β remains to be determined, but a possibility is that it may be secondary to increased transmission of systemic pressure to the glomerular capillary pressure because glomerular mesangial cells and podocytes are known to respond to increases in cyclic stretch, at least in vitro.29
protein synthesis, the synthesis of basement membrane, or effects of TGF-β on epithelial or mesangial cell proliferation and survival. Possible mechanisms by which TGF-β may directly alter $P_{\text{m}}$ include contraction or cytoskeletal changes that alter the shape of podocytes or capillary endothelial cells or phosphorylation or dephosphorylation of the junctional complexes between slit pores or the adhesion molecules anchoring the foot processes to the glomerular basement membrane.

Previous studies have indicated that elevations in the renal formation of 20-HETE reduce the degree of renal injury and proteinuria during hypertension development in Dahl S rats. Moreover, McCarthy et al reported recently that 20-HETE has a protective action on the glomerulus to prevent changes in $P_{\text{m}}$ induced by focal segmental glomerulosclerosis factor. Thus, we examined whether TGF-β might increase $P_{\text{m}}$ by inhibiting the glomerular production of 20-HETE. The results indicate that isolated glomeruli avidly produce 20-HETE, other HETEs, and EETs when incubated with AA and that TGF-β selectively inhibits formation of 20-HETE. In further experiments, we found that preventing the fall in 20-HETE levels by adding a stable 20-HETE agonist (W1003) opposed the increase in $P_{\text{m}}$ produced by TGF-β. These studies indicate that a fall in the glomerular production of 20-HETE contributes to the increase in $P_{\text{m}}$ produced by TGF-β. The mechanism by which TGF-β inhibits the formation of 20-HETE remains to be determined. A possibility is that TGF-β may stimulate production of NO and superoxide radicals in the glomerulus because both of these compounds have been shown recently to inhibit formation of 20-HETE by binding to heme in CYP4A enzymes.

**Perspective**

Previous studies have indicated that TGF-β levels are elevated in the kidney in hypertension and diabetes and that TGF-β plays a critical role in development of glomerulosclerosis and fibrosis. However, the mechanism by which TGF-β initiates development of proteinuria and renal injury has remained elusive. The results of the present study indicate that TGF-β inhibits production of 20-HETE in the glomerulus and that this leads to an increase in the permeability and filtration of macromolecules and growth factors into tubular fluid. Exposure of glomerular and tubular epithelial cells to albumin and growth factors has been shown to induce the synthesis of TGF-β leading to epithelial–mesenchymal transformation, increased formation of extracellular matrix, and development of glomerulosclerosis and renal interstitial fibrosis. This mechanism may help explain how elevations in glomerular capillary pressure or glomerular hyperfiltration may increase production of glomerular TGF-β, which then contributes to development of proteinuria, glomerulosclerosis, and renal interstitial in hypertension, diabetes, and other models of renal injury and fibrosis. The present results also suggest that administration of 20-HETE agonists may be glomeruloprotective and oppose the development of hypertension and diabetic nephropathy and other glomerular diseases associated with hyperfiltration and elevations in the renal production of TGF-β.

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**References**


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