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

Annette J. Dahly-Vernon, Mukut Sharma, Ellen T. McCarthy, Virginia J. Savin, Steven R. Ledbetter, Richard J. Roman

Abstract—This study examined the role of transforming growth factor-β (TGF-β) in altering the glomerular permeability to albumin (P_{ab}) 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 P_{ab} 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 P_{ab} 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 P_{ab} 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 P_{ab} and opposed the effects of TGF-β to increase P_{ab}. 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 P_{ab} through inhibition of the glomerular production of 20-HETE. (Hypertension. 2005;45[part 2]:643-648.)

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

Dahl salt-sensitive (Dahl S) rats exhibit many traits associated with salt-sensitive hypertension in humans. They are salt sensitive, insulin resistant, and hyperlipidemic, and they rapidly develop proteinuria and glomerulosclerosis when challenged with a high-salt (HS) diet. The glomerular lesions that develop resemble those seen in patients with hypertension- and diabetes-induced nephropathy. 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 rats fed an HS diet and that chronic treatment of Dahl S rats with a TGF-β neutralizing antibody (Ab) reduces proteinuria and the degree of glomerulosclerosis and fibrosis. Previous studies on the role of TGF-β in the pathogenesis of renal disease have focused on its effects to induce the expression of genes involved in formation of extracellular matrix. However, TGF-β has been reported recently to directly increase the permeability of isolated glomeruli to albumin. Damage to the glomerular filtration barrier and increased filtration of macromolecules or growth factors stimulate podocytes, which increases the production of extracellular matrix and promotes the development of glomerulosclerosis and renal interstitial fibrosis. However, the mechanism by which TGF-β increases the glomerular permeability to albumin (P_{ab}) is unknown. In view of the recent findings that induction of the renal formation of 20-HETE with fibrates or introgression of the cytochrome P450 4A (CYP4A) region of chromosome 5 from Lewis rats into a congenic strain of Dahl S rats reduces proteinuria, glomerulosclerosis, and tubulointerstitial fibrosis, the present study examined the role of TGF-β in altering the glomerular P_{ab} 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 proteinuria and P_{ab} during hypertension development...
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 Pab 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 Pab
Glomeruli were isolated using the sieving method as described previously in a media containing 5 g/dL of BSA. In each experimental condition, Pab was determined from the change in glomerular volume (ΔV) after exchange of the bath with medium containing 1 g/dL albumin. Pab was calculated as 1 - (ΔVexperimental/ΔVcontrol), 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 Pab 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 Pab.

In other experiments, we examined the interaction of TGF-β and 20-HETE on Pab 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-β (10 ng/mL) for 15 minutes at 37°C, and changes in Pab were determined. Glomeruli were also pretreated with a stable 20-HETE agonist, 20-hydroxyeicosatetraenoic acid, adjusted to a concentration of 10 ng of internal standard, 14,15-epoxyeicosa-5(Z)-enoic-methyl sulfonylethyl (EEZE; m/z 323) was determined and compared with a standard curve constructed with 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 isotropic 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 EETs 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 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 ANOVA followed by 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 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 sulfonylethyl (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 isotropic 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 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.

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Results
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Figure 1. Expression of TGF-β1 in the kidney of Sprague Dawley and Dahl S rats fed an LS and HS for 7 days. Renal homogenates isolated from Sprague Dawley (lanes 1 through 3), Dahl S rats fed an LS diet (lanes 4 through 7), and Dahl S rats fed an HS diet (8% NaCl) for 7 days (lanes 8 through 11). Each lane was loaded with a homogenate (30 μg protein per lane) isolated from different animals (n = 3 to 4 per group). Significant difference vs the values seen in Dahl S rats fed an LS diet; SD, Sprague Dawley; HS-7, HS diet for 7 days.
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_{alb}**
A comparison of P_{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_{alb} was significantly higher in Dahl S rats maintained on an LS diet than in control Sprague Dawley rats. P_{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_{alb} in Dahl S rats fed an HS diet for 1 week was associated with a significant rise in blood pressure from 121/110 to 136/113 mmHg (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-β in Altering P_{alb} in Dahl S Rats**
A comparison of the effects of exogenous administration of TGF-β1 (10 ng/mL) on P_{alb} in glomeruli isolated from Sprague Dawley and Dahl S rats is also summarized in Figure 2. TGF-β1 increased P_{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-β1 also increased P_{alb} in Dahl S rats fed an HS diet for 4 days, but it had no effect on P_{alb} in Dahl S rats fed an HS diet for 7 days because the baseline P_{alb} in these rats was already near maximal.

Chronic treatment of Dahl S rats fed an HS diet with a TGF-β neutralizing Ab prevented the increase in baseline P_{alb}. Administration of TGF-β1 to these glomeruli still increased P_{alb}, similar to that seen in glomeruli isolated from control Sprague Dawley rats and Dahl S rats fed an LS diet. TGF-β 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-β 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-β Ab (Figure 3C).

**Effect of TGF-β on Glomerular Production of 20-HETE**
The effects of TGF-β 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
Effects of a 20-HETE Agonist on P_{alb}

The effect of addition of a 20-HETE agonist on the changes in P_{alb} produced by TGF-β1 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} produced by TGF-β1. Similar results were obtained with Dahl S rats maintained on an LS diet or 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} of glomeruli early in hypertension development in Dahl S rats and whether TGF-β may act in part by inhibiting glomerular production of 20-HETE. The results indicate that baseline P_{alb} increases markedly in Dahl S rats fed an HS diet for only 4 days, and it reaches a maximum after 7 days on this diet. The 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, 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.

The present study also explored the mechanism by which TGF-β may increase P_{alb} of the glomerulus. We confirmed previous findings of Sharma et al. that TGF-β1 increases P_{alb} of glomeruli isolated from Sprague Dawley rats. The rapid nature of this response (5 to 15 minutes) indicates that it is mediated by a direct signaling event distinct from the longer-term responses to TGF-β that alter gene expression and
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\textsubscript{GFR} 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.\textsuperscript{31} Moreover, McCarthy et al\textsuperscript{32} reported recently that 20-HETE has a protective action on the glomerulus to prevent changes in P\textsubscript{GFR} induced by focal segmental glomerulosclerosis factor. Thus, we examined whether TGF-β might increase P\textsubscript{GFR} by inhibiting the glomerular production of 20-HETE. The results indicate that isolated glomeruli avidly produce 20-HETE, inhibiting the glomerular production of 20-HETE. The results of the present study indicate that isolated glomeruli avidly produce 20-HETE, other HETEs, and EETs when incubated with AA and that TGF-β1 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 (WIT003) opposed the increase in P\textsubscript{GFR} produced by TGF-β1. These studies indicate that a fall in the glomerular production of 20-HETE contributes to the increase in P\textsubscript{GFR} produced by TGF-β1. 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\textsuperscript{16,33–35} 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.\textsuperscript{36–38}

**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.\textsuperscript{15,21,22,30,39,46} 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.\textsuperscript{22,41,42} 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.


