TGF-β impairs renal autoregulation via generation of ROS

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Impaired autoregulation in chronic kidney disease can result in elevation of glomerular capillary pressure and progressive glomerular damage; however, the factors linking chronic glomerular disorders to impaired autoregulation have not been identified. We tested the hypothesis that the cytokine most closely associated with progressive glomerular disease, transforming growth factor (TGF)-β, may also attenuate autoregulation.

METHODS

Animals. One hundred sixty male Sprague-Dawley rats (250–400 g) were used for the autoregulatory experiments, in situ studies, and for the isolation of renal microvascular smooth muscle cells. Studies were approved by the Committees on Animal Use for Research and Education at the Medical College of Georgia and by the IACUC committee at Thomas Jefferson University.

Kidney preparation. In vitro videomicroscopy experiments were conducted using the blood-perfused juxtamedullary nephron technique, as previously described (17, 19). After the microdissection procedures to prepare the kidney for study, the kidneys were perfused with blood collected and prepared from donor rats. The blood perfusion pressure was continuously monitored using a pressure cannula positioned in the tip of the juxtamedullary afferent arteriole with tempol, a ROS scavenger, or with apocynin, a NADPH oxidase inhibitor, prevented the impaired autoregulatory responses were measured under control conditions and during superfusion with TGF-β1 (10 ng/ml). Control afferent arteriolar diameter averaged 18.4 ± 1 μm and significantly decreased to 16.3 ± 0.9 and 13.2 ± 0.8 μm at perfusion pressures of 130 and 160 mmHg, respectively. In the presence of TGF-β1, autoregulatory responses were completely blocked. In similar experiments performed using PDGF-BB (10 ng/ml) and HGF (25 ng/ml), the normal autoregulatory response was not affected. In vitro studies, using isolated preglomerular vascular smooth muscle cells, revealed that exposure to TGF-β1 stimulated a rapid increase in reactive oxygen species (ROS) that was inhibited by NADPH oxidase inhibitors. In situ studies, with dithiothreitol staining, revealed a marked increase in renal vessel ROS production on exposure to TGF-β1. Pretreatment of the juxtamedullary afferent arterioles with tempol, a ROS scavenger, or with apocynin, a NADPH oxidase inhibitor, prevented the impaired autoregulation induced by TGF-β1. These data reveal a novel hemodynamic pathway by which TGF-β could lead to progressive glomerular injury by impairing normal renal microvascular function.

The basis for glomerular hypertrophy has been attributed to several factors; however, a major contributor in the initial development of glomerular volume increase is due to diabetic glomerular hypertension. During the development of diabetic glomerular hypertrophy, an increase in glomerular capillary pressure appears to be primarily due to inappropriate dilation of the afferent arteriole and consequent increase in glomerular blood flow (15). This increase in glomerular capillary pressure is considered a key factor in increasing glomerular distension, leading to mesangial matrix production, capillary endothelial cell dysfunction, and podocyte stretching with consequent podocyte dropout and glomerular sclerosis (24).

An explanation for the elevation in glomerular capillary pressure may be impairment of autoregulation by afferent arterioles. Normally, afferent arterioles respond to an increase or decrease in arterial pressure with vasoconstriction or vasorelaxation, respectively. These resistance adjustments serve to maintain a relatively constant glomerular capillary pressure and glomerular filtration rate (16). In several disease states such as diabetic nephropathy, hypertension, subtotal nephrectomy, and focal sclerosis, there is loss of autoregulatory control with consequent elevation of glomerular capillary pressure (4, 16, 24, 33). However, the factors that link these various forms of progressive glomerular disease with loss of autoregulation have not been identified (14). As the prosclerotic factor transforming growth factor (TGF)-β has previously been found to be upregulated in glomeruli of all progressive glomerulopathies (47) and is known to mediate glomerular enlargement in early diabetes (39, 40), we evaluated the hypothesis that TGF-β is able to influence autoregulatory behavior.

THE INCIDENCE OF END-STAGE renal disease is rapidly approaching epidemic proportions in the United States. Much of the cause of this rise is due to diabetic nephropathy and hypertension in susceptible risk groups (30a). Patients with a tendency to develop progressive renal disease appear to share the common finding of glomerular hypertrophy before the onset of severe renal disease (1). The finding of glomerular hypertrophy has been recognized in patients with type 1 and type 2 diabetes (25, 32), Pima Indians (35), and in patients with essential hypertension (22).

The basis for glomerular hypertrophy has been attributed to several factors; however, a major contributor in the initial development of glomerular volume increase is an increase in renal hemodynamics; kidney disease; hypertension; growth factors; oxidative stress; NADPH oxidase
Quincy, MA). Perfusion pressure was initially set at 100 mmHg for control measurements and increased to 130 and 160 mmHg by increasing the reservoir pressure. The inner cortical surface of the kidney was continuously superfused with warmed (37°C) Tyrode’s buffer containing 10.0 g/l BSA, and the kidney was allowed to equilibrate for at least 15 min. Afferent arteriolar responses to changes in renal perfusion pressure (RPP) or administration of vasoactive agonists and antagonists were determined, as previously described (17, 19). Autoregulatory behavior was assessed by measuring changes in afferent arteriolar diameter in response to acute elevations in RPP. Measurements of afferent arteriolar diameter were made at a single site, at 12-s intervals. Sustained afferent arteriolar diameters were calculated from the average of measurements made during the final 2 min of each treatment period. Each protocol began with a 5-min control period to ensure a stable vessel diameter and was followed by either agonist stimulation or an increase in RPP, to establish the control response.

Effect of TGF-β1, PDGF, and HGF on the afferent arteriolar response to acute increases in RPP. The effect of increasing RPP on afferent arteriolar diameter was determined before and during exposure to TGF-β1, PDGF-BB, or HGF (R&D Systems, Minneapolis, MN). Afferent arteriolar diameters were measured at perfusion pressures of 100, 130, 160, and again at 100 mmHg, in successive 5-min periods. After recovery, the superfusion solution was changed to a similar solution containing TGF-β1 (1, 5, 7.5, or 10 ng/ml), PDGF-BB (10 ng/ml), or HGF (25 ng/ml), and pressure-induced responses were reassessed. Finally, the superfusion solution was changed to a similar solution without growth factors and pressure-induced responses were assessed again. Vehicle control studies were also performed.

To determine the effect of TGF-β1 on regional afferent arteriolar responses, studies were performed to measure proximal, midportion, and terminal regions of the afferent arteriole. Afferent arteriolar diameters were measured at perfusion pressures of 100, 130, 160, and again at 100 mmHg. At each perfusion pressure, the diameter of the arteriole was determined at a site near the origin of the afferent arteriole, a site near the midpoint of the arteriole, and finally at a site adjacent to the arteriolar attachment to the glomerulus. After recovery, the superfusion solution was changed to a similar solution containing TGF-β1 (10 ng/ml), and pressure-induced responses were reassessed at the same locations.

Effect of TGF-β1 on the afferent arteriolar response to vasoactive agonists. The effect of TGF-β1 on the afferent arteriolar response to vasoconstrictor agonists was assessed using ATP (10 μM), adenosine (Ado; 10 μM), KCl (55 mM), and angiotensin II (ANG II; 1 nM, Sigma, St. Louis, MO). After a baseline diameter at 100 mmHg was established, control vasoconstrictor responses were obtained. After recovery, the superfusion solution was changed to a similar solution containing TGF-β1 (10 ng/ml), and pressure-induced responses were reassessed at the same locations.

Effect of tempol and apocynin on TGF-β1-mediated attenuation of afferent arteriolar responsiveness. The effects of the superoxide scavenger tempol (Sigma) and a NADPH oxidase inhibitor, apocynin (Aldrich Chemical, Milwaukee, WI), were determined on basal and TGF-β1-mediated impairment of afferent arteriolar autoregulatory behavior. Afferent arteriolar diameters were measured at perfusion pressures of 100, 130, 160, and again at 100 mmHg, in successive 5-min periods. After recovery, the superfusion solution was changed to a similar solution containing tempol (1 mM) or apocynin (100 μM). After 20 min, TGF-β1 (10 ng/ml) was added to the superfusate and pressure-induced responses were reassessed. Apocynin was initially dissolved in ethanol before being diluted in the 1% albumin superfusate solution.

Renal microvessel smooth muscle cell isolation and reactive oxygen species measurement. Preglomerular smooth muscle cells were prepared from normal rats, as previously described (20). Freshly isolated preglomerular smooth muscle cells were plated directly on polylysine-coated coverslips and characterized by immunofluorescence for α-smooth muscle actin and heavy chain myosin, as previously described (39). As an index of reactive oxygen species (ROS) generation, the coverslips were loaded for 30 min at 37°C with 0.2 mM 5-(and-6)-chloromethyl-2, 7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA, Molecular Probes, Eugene, OR). TGF-β1 (10 ng/ml) was added to the coverslips, and ROS generation was monitored in live cells using confocal microscopy. In parallel experiments, tempol (1 mM), diphenyleneiodonium chloride (DPI; 1 μM, Sigma), or apocynin (100 μM, Aldrich Chemical) was added to separate wells for 20–60 min before addition of TGF-β1.

In situ detection of superoxide in renal tissue. Hydroethidine, an oxidative fluorescent dye, has been used to evaluate levels of superoxide in situ (28). Kidneys were perfused with PBS and quickly removed from the rat. The upper pole of the kidney was removed, and vertical sections, of 1-mm thickness, were prepared and placed in a six-well tissue culture plate containing DMEM/10% FCS at 37°C. Three individual slices were treated with TGF-β1 (10 ng/ml, 5 min). Three other slices were left untreated to serve as paired controls. Subsequently, the tissue slices were embedded in OCT and snap-frozen in liquid nitrogen. After 2 h at −70°C, 30-μm-thick sections were cut and placed on glass slides. The slides were incubated with 10 μM dihydroethidium (DHE) in DMSO for 30 min at 37°C. Slides were examined and photographed using immunofluorescence microscopy.

Statistical analysis. Data from microvascular studies were evaluated using one-way analysis of variance for repeated measures. Differences between group means, within each series, were determined using the Newman-Keuls multiple range test. Dose-response data were analyzed across groups by one-way analysis of variance combined with the Newman-Keuls multiple range test. P values <0.05 were considered to indicate statistically significant differences. All values are reported as means ± SE.

RESULTS

TGF-β inhibits the autoregulatory response. Initial experiments determined the effect of TGF-β on pressure-mediated autoregulatory responses (Fig. 1). Under control conditions, at 100 mmHg, afferent arteriolar diameter averaged 17.7 ± 1.1 μm and decreased by 11 ± 1 and 27 ± 1% (P < 0.05 vs. control) when RPP was increased to 130 and 160 mmHg, respectively. Returning RPP to 100 mmHg resulted in a complete recovery to 17.4 ± 1.1 μm. Addition of 10 ng/ml TGF-β1 to the superfusate did not change baseline caliber as the arteriolar diameter averaged 18.1 ± 0.9 μm. However, pressure-mediated autoregulatory responses were completely blocked.

Fig. 1. Effect of transforming growth factor (TGF)-β1 on the autoregulatory response of juxtamedullary afferent arterioles. Each data point represents the mean diameter measured at 12-s intervals. Control responses are depicted by the open symbols, responses during exposure to 10 ng/ml TGF-β are shown by the filled symbols, and the response after the recovery period is shown by the gray symbols; n = 6 arterioles. *P < 0.05 vs. control diameter.
during exposure (15 min) to TGF-β1. Increasing RPP from 100 to 130 and 160 mmHg resulted in arteriolar diameters of 18.0 ± 0.9 and 18.0 ± 1.0 μm, respectively. Washout (15 min) of TGF-β1 from the kidney surface restored significant autoregulatory responses. Step increases in perfusion pressure to 130 and 160 mmHg reduced arteriolar diameter by 7 ± 2 (P < 0.05) and 13 ± 2% (P < 0.05). A dose-response relationship to TGF-β1 was established. As shown in Fig. 2, exposure of kidneys to 1.0 ng/ml TGF-β1 had no detectable effect on the autoregulatory response, whereas a concentration of 7.5 ng/ml TGF-β1 (n = 5) produced a significant attenuation of autoregulatory responsiveness.

**PDGF and HGF do not inhibit autoregulation.** To ensure that the results described above were specific to TGF-β1, similar studies were performed with two other growth factors, PDGF and HGF (Fig. 3). Exposure of afferent arterioles to PDGF (10 ng/ml; Fig. 3A) or HGF (25 ng/ml; Fig. 3B) had no detectable effect on the autoregulatory response. In the PDGF group, pressure-mediated vasoconstriction averaged 11 ± 1 (P < 0.05) and 28 ± 3% (P < 0.05) under control conditions and 15 ± 2 (P < 0.05) and 27 ± 3% (P < 0.05) in the presence of PDGF. Similarly, in the HGF group, control responses averaged 13 ± 1 (P < 0.05) and 26 ± 3% (P < 0.05), whereas in the presence of HGF responses averaged 11 ± 2 (P < 0.05) and 24 ± 4% (P < 0.05).

**TGF-β1 impairs autoregulatory behavior in both myogenic- and tubuloglomerular feedback-sensitive regions of the afferent arteriole.** Autoregulatory control is accomplished through the combined influences of both the myogenic and tubuloglomerular feedback mechanisms. The terminal aspect of the afferent arteriole is purportedly controlled by tubuloglomerular feedback influences, whereas regulation of the early portion of the arteriole involves myogenic control. To determine whether TGF-β1 preferentially inhibits tubuloglomerular feedback or myogenic reactivity, pressure-mediated vasoconstriction was assessed at proximal, mid, and distal sites along the arteriole under control conditions, and again during exposure to TGF-β1 (Fig. 4). Arterioles in this group averaged 826 ± 167 μm in length (n = 6), and measurements made midway along the arteriole averaged 403 ± 81 μm from the glomerulus. Proximal measurements were made 758 ± 159 μm from the glomerulus, and distal measurements averaged 51 ± 4 μm from the glomerulus. Under control conditions, the magnitude of the vasoconstriction evoked by increasing perfusion pressure was similar at all three sites. In response to increasing perfusion pressure to 160 mmHg, afferent arteriolar diameter declined similarly by 12 ± 1, 17 ± 3, and 13 ± 2% at the proximal, mid, and distal arteriole sites, respectively (Fig. 4, left). Subsequent introduction of TGF-β1 to the superfuse did not alter baseline diameter at any of the sites along the arteriole; however, it significantly impaired autoregulatory responses at the proximal, mid, and distal sites, respectively (Fig. 4, right).

**TGF-β does not inhibit mediators or modulators of autoregulatory behavior.** Experiments were performed to determine whether TGF-β1 impairs autoregulatory behavior by interfering with the actions of purported mediators of tubuloglomerular feedback and autoregulatory responses or by generally interfering with vasoconstrictor mechanisms. Accordingly, we examined the effect of TGF-β1 on the afferent arteriolar response to adenosine, ATP, ANG II, and KCl. As shown in Fig. 5, TGF-β1 treatment had no detectable effect on the magnitude, or time course, of the vasoconstriction produced by any of these agents. There was no difference between control and TGF-β1-treated preparations under basal conditions or with exposure to 10 μM adenosine (Fig. 5A). This implies that TGF-β1 does not interfere with adenosine A1 receptors or the intracellular signaling mechanisms employed by adenosine. Similarly, preexposure to TGF-β1 did not alter the vasoconstrictor response to ATP (Fig. 5B), suggesting normal purinoceptor signaling and vascular smooth muscle cell function.

Vasoconstrictor responses evoked by KCl and ANG II were nearly identical before and during exposure to TGF-β1 (Fig. 5, AJP-Renal Physiol • VOL 288 • MAY 2005 • www.ajprenal.org
As afferent arteriolar vasoconstriction by these agents is largely dependent on functional L-type calcium channels (16), these data indicate that voltage-dependent calcium channel function remains intact in the presence of TGF-β.

**Regulation of ROS by TGF-β in renovascular cells and tissue.** To identify the mechanisms by which TGF-β may interfere with the autoregulatory response, we isolated preglomerular vascular smooth muscle cells and examined the direct effects of TGF-β1. As oxygen radicals have been linked to regulation of vascular reactivity, studies were focused on the possible effect of TGF-β1 to generate ROS. TGF-β1 stimulated an increase in DCF fluorescence in preglomerular smooth muscle cells within 5 min of exposure (Fig. 6A). TGF-β1-mediated stimulation of ROS was completely inhibited in cells that were pretreated with tempol, a membrane-permeable SOD mimic. Additionally, ROS generation by TGF-β1 was inhibited by apocynin, a NADPH oxidase inhibitor (Fig. 6B) and DPI (data not shown).

To determine whether a similar response would occur in the rat kidney, ROS production was measured in kidney tissue slices under control conditions and in response to TGF-β (Fig. 6C). DHE staining of thick sections revealed that exposure to TGF-β1 stimulated ROS production in renal vessels. The increase in ROS levels was observed in endothelial cells, media, and adventitia. Using this technique, it is difficult to definitively establish the identity of smaller vessels in the kidney.

**Role of ROS in TGF-β-mediated regulation of autoregulation.** To evaluate the role of TGF-β-stimulated generation of ROS, we examined the effect of tempol on TGF-β1-mediated impairment of autoregulatory behavior. Autoregulatory responses were examined before and during TGF-β1 treatment; however, each kidney was preincubated with tempol (1.0 mM) for 20 min. As shown in Fig. 7A, tempol pretreatment completely prevented TGF-β1-mediated attenuation of the autoregulatory response. Under these conditions, pressure-mediated autoregulatory responses were normalized compared with results with TGF-β1 alone. Increasing RPP from 100 to 130 and 160 mmHg resulted in arteriolar diameters 13 ± 4 and 23 ± 9% smaller than the control diameter, respectively. These changes are not significantly different from the changes observed in the control period. Similarly prepared time control studies indicate that tempol alone does not alter normal autoregulatory responses (Fig. 7B).

To determine the involvement of the NADPH oxidase system in the TGF-β1-mediated impairment of autoregulation, we examined the effect of pretreatment with the NADPH oxidase inhibitor apocynin on the ability of TGF-β1 to attenuate autoregulatory responsiveness (Fig. 8). Similar to tempol, apocynin (100 μM) pretreatment completely restored the normal autoregulatory response despite exposure to TGF-β. In addition, apocynin alone did not significantly alter autoregulatory behavior (data not shown).

**DISCUSSION**

In the present study, we find that the normal preglomerular autoregulatory response is markedly attenuated during exposure to TGF-β1. This effect of TGF-β is specific and concentration dependent. NADPH oxidase-dependent stimulation of ROS in preglomerular vascular smooth muscle cells appears to be a key pathway by which TGF-β1 elicits its effects.

TGF-β has been implicated in a variety of progressive glomerulopathies, primarily because of its prosclerotic profile. TGF-β promotes expansion of extracellular matrix in glomeruli via stimulation of extracellular matrix deposition and inhibition of matrix degradation (3, 48). However, the role of TGF-β in mediating vascular alterations in disease states has not been widely appreciated. We have found that TGF-β is upregulated and mediates diabetic glomerular enlargement in both rat and mouse models of diabetes (39, 40). In the setting of early diabetes in the rat, antibodies against TGF-β attenuated the glomerular volume increase primarily by decreasing capillary loop distension (39). The results of the present study expand the vascular role of TGF-β by establishing its ability to inhibit autoregulatory behavior of preglomerular arterioles.

The concentration of TGF-β required to inhibit the normal autoregulatory behavior is between 5 and 10 ng/ml (200–400 pM). In a normal, healthy kidney, this concentration of TGF-β1 may not be achieved in the perivascular microenvironment,
although it is extremely difficult to accurately measure levels of bioactive factors in discrete microenvironments. However, in pathophysiological states, where renal TGF-\(\beta\) production is markedly increased, higher perivascular concentrations of TGF-\(\beta\) may approach these levels. In previous studies, we demonstrated that local production of TGF-\(\beta\) is 5- to 10-fold higher in diseased glomeruli from diabetic rats and renal cortex from cyclosporine-treated rats than in their matched controls (21, 39). Administration of anti-TGF-\(\beta\) antibody ameliorated the renal injury associated with elevation of intrarenal TGF-\(\beta\) in diabetic rats, cyclosporine-treated rats, and hypertensive Dahl salt-sensitive rats (5, 21, 39). Given that TGF-\(\beta_1\) concentrations may reach levels of 5–10 ng/ml (200–400 pM) in normal plasma and 50–100 ng/ml (2–4 nM) in serum, the concentrations used in the current report are likely within the biological range that might be expected in disease states, such as diabetes or hypertension. Several prior studies have used concentrations of TGF-\(\beta_1\) between 5 and 20 ng/ml to demonstrate proof of this concept, especially in local tissue-type preparations (2, 6, 8). Furthermore, human and rat vascular smooth muscle cells avidly secrete high levels (5–13 ng/ml) of active TGF-\(\beta\) into conditioned medium (12, 23). It is also important to note that in the juxtamedullary nephron preparation, the superfusate will be exposed to blood from the perfusate; thus the active TGF-\(\beta_1\) present in the superfusate may bind to a variety of plasma proteins (11, 42) present in circulating blood. Therefore, it is likely that the interstitial fluid level of active TGF-\(\beta_1\) in the perivascular area is lower than the superfusate levels of TGF-\(\beta_1\). Finally, TGF-\(\beta\) isoforms and TGF-\(\beta\) receptors have been localized to the juxtaglomerular apparatus and to afferent arteriolar smooth muscle cells (13, 34) and are increased in progressive glomerular diseases (47). We therefore postulate that excess local production of TGF-\(\beta\) isoforms, or their receptors, is accompanied by exaggerated paracrine interaction between interstitial TGF-\(\beta\) and afferent arteriolar smooth muscle cells, leading to reduction of autoregulatory responsiveness during disease states.

Other candidate growth factors that have been implicated in chronic glomerular disease and may play a role in impaired autoregulation include PDGF, HGF, and IGF. PDGF-\(\beta\) chain is upregulated in glomeruli of animal models associated with impaired renal autoregulation, including diabetes (30) and focal glomerulosclerosis (41). Although PDGF may contribute to glomerular disease via multiple pathways, we did not find an effect of PDGF-BB on the autoregulatory capability of afferent arterioles. In addition, although PDGF has been reported to stimulate ROS in isolated vascular smooth muscle cells, we found no evidence of ROS production by PDGF in preglomerular vessels (data not shown). Of note, the dose of PDGF-BB to maximally stimulate ROS production is in the range of 30–100 ng/ml (29), and it is possible that higher concentrations of PDGF may also affect autoregulation. HGF is increased in diabetic nephropathy and may contribute to, or ameliorate, renal disease (27, 44). Based on our results, these effects of HGF appear to be independent of modulating the autoregulatory response. IGF has a complex pattern of regulation in diabetic kidney disease (9, 10). Interestingly, IGF-1 improved afferent arteriolar autoregulation in the model of 5/6 nephrectomy (26). Therefore, it is unlikely that IGF would impair autoregulation in the model used in the current report.
One possible explanation for impairment of autoregulatory responses is that acute exposure to TGF-β1 induces a general disruption of the contractile apparatus needed for contraction of vascular smooth muscle. Alternatively, TGF-β could be stimulating excessive production of vasodilatory influences that offset pressure-induced, autoregulatory vasoconstrictor signals. Examination of the baseline diameters, before and during TGF-β1 treatment, demonstrates that TGF-β1 alone has no effect on ambient arteriolar diameter. These data suggest that TGF-β1 is not stimulating significant levels of vasodilator production that would tend to increase afferent arteriolar diameter or attenuate agonist-mediated vasoconstrictor responses. A second line of evidence comes from the studies evaluating the vasoconstrictor responses to adenosine, ATP,
KCl-induced depolarization. These results suggest that TGF-β promotes voltage-dependent vasoconstriction of afferent arterioles by modulators of autoregulatory and tubuloglomerular feedback. The present study, we demonstrated that renal vessels and preglomerular vascular smooth muscle cells respond to TGF-β1 with an increase in ROS generation. Using DHE staining, we found clear evidence of ROS production in renal vessels, on short-term TGF-β1 stimulation. Isolated smooth muscle cells, derived from the same vascular segments that affect autoregulatory adjustments in renal vascular resistance, also exhibit ROS stimulation in response to TGF-β1. The time course by which TGF-β1 induced ROS generation in renal vessels and smooth muscle cells was consistent with the effect of TGF-β on the afferent arteriole in the isolated kidney preparation. Furthermore, the superoxide scavenger tempol completely inhibited ROS accumulation in freshly isolated preglomerular smooth muscle cells and restored normal autoregulatory behavior in the presence of TGF-β1.

Stimulation of ROS by TGF-β1 likely involves NADPH oxidase, as other pathways for ROS generation such as mitochondrial respiration, xanthine oxidase, arachidonate-derived enzymes, and nitric oxide synthase are relatively minor sources of ROS in vascular smooth muscle cells (37). In support of the involvement of NADPH oxidase, the flavoprotein inhibitor DPI and the NADPH oxidase inhibitor apocynin inhibited ROS accumulation in freshly isolated preglomerular smooth muscle cells. Apocynin also restored normal autoregulatory responses in the presence of TGF-β1. Collectively, these data strongly implicate superoxide generation, by NADPH oxidase, as a primary mechanism in ROS-mediated impairment of autoregulatory behavior by TGF-β1. It is interesting to note that neither tempol nor apocynin altered basal afferent arteriolar diameter or interfered with normal autoregulatory responses. These data would suggest that basal production of reactive oxygen radicals does not have an important role in maintaining basal vascular tone, nor in the autoregulatory response under basal conditions. Our data are consistent with a prior report that ROS scavenging by tempol, or inhibition of superoxide production by apocynin, had no effect on basal tone of the afferent arteriole in rabbits (37). However, several studies have implicated ROS production in mediating or enhancing vasoconstrictor activity in afferent arterioles under conditions of direct exposure to vasoconstrictors, or in response to tubuloglomerular feedback (36, 38, 46). ROS production reportedly enhances vasoconstriction by inhibiting nitric oxide action (36, 38, 46) and affecting the phosphorylation status of myosin light chains (7). Although ROS production may play a role to enhance

![Figure 7](http://example.com/fig7.png)

**Fig. 7.** Scavenging of superoxide blocks the TGF-β1-induced inhibition of autoregulatory responses. A: pressure-induced changes in afferent arteriolar diameter are shown before (open symbols) and during (filled symbols) incubation with tempol (1 mM) and TGF-β1 (10 ng/ml); n = 6 arterioles. *P < 0.05 vs. control diameter. B: effect of tempol alone on autoregulatory responsiveness. Pressure-induced changes in afferent arteriolar diameter are shown before (open symbols) and during (filled symbols) incubation with tempol (1 mM); n = 5 arterioles. *P < 0.05 vs. control diameter.

ANG II, or KCl. In each case, the time course and magnitude of the vasosconstriction evoked by each agent were unchanged by the presence of TGF-β1. This observation demonstrates that the contractile apparatus of afferent arteriole smooth muscle remains intact and unaffected by TGF-β treatment.

Renal autoregulation is accomplished by the combined influences of the myogenic pathway and tubuloglomerular feedback. Interestingly, the inhibitory effect of TGF-β on autoregulatory behavior does not appear to discriminate between these two components. Pressure-mediated vasoconstriction was uniformly attenuated along the entire length of the afferent arterioles studied. TGF-β did not interfere with vasoconstriction by ATP, adenosine, or ANG II, which are purported mediators and modulators of autoregulatory and tubuloglomerular feedback responses. Furthermore, TGF-β did not interfere with voltage-dependent vasoconstriction of afferent arterioles by KCl-induced depolarization. These results suggest that TGF-β may specifically uncouple mechanosensitive pathways involved in myogenic and tubuloglomerular feedback responses, without affecting receptors and signaling pathways for adenosine, ATP, ANG II, and voltage-gated vasoconstriction (16).

The mechanism by which TGF-β attenuates autoregulatory behavior appears to involve local production of ROS. In the present study, we demonstrated that renal vessels and preglomerular vascular smooth muscle cells respond to TGF-β1 with

![Figure 8](http://example.com/fig8.png)

**Fig. 8.** NADPH oxidase inhibition blocks the TGF-β1-induced inhibition of autoregulatory responses. Pressure-induced changes in afferent arteriolar diameter are shown before (open symbols) and during (filled symbols) incubation with apocynin (100 μM) and TGF-β1 (10 ng/ml); n = 5 arterioles. *P < 0.05 vs. control diameter. #P < 0.05 vs. apocynin control diameter.
vascular reactivity to vasoconstrictors, our results suggest that, under the cover of excess TGF-β exposure, increased ROS production mediates the uncoupling of afferent arteriolar constriction in response to increased perfusion pressure.

In summary, we have demonstrated that the proscerotocytic cytokine TGF-β1 is capable of inhibiting the normal, pressure-mediated renal autoregulatory response. The pathway involves ROS generation in renal microvascular smooth muscle cells and is blocked by superoxide scavengers and NADPH oxidase inhibitors. These studies therefore establish prima facie evidence that TGF-β may be a critical factor contributing to impaired autoregulation in states of chronic glomerular disease.

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