The Angiotensin Type 1 Receptor Antagonist, Eprosartan, Attenuates the Progression of Renal Disease in Spontaneously Hypertensive Stroke-Prone Rats with Accelerated Hypertension

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ABSTRACT

The effects of the angiotensin type 1 (AT₁) receptor antagonist, eprosartan, were studied in a model of severe, chronic hypertension. Treatment of male spontaneously hypertensive stroke prone rats (SHR-SP) fed a high-fat, high-salt diet with eprosartan (60 mg/kg/day i.p.) for 12 weeks resulted in a lowering of blood pressure (250 ± 9 versus 284 ± 8 mm Hg), renal expression of transforming growth factor-β mRNA (1.5 ± 0.2 versus 5.4 ± 1.4) and the matrix components: plasminogen activator inhibitor-1 (5.2 ± 1.4 versus 31.4 ± 10.7), fibronectin (2.2 ± 0.6 versus 8.2 ± 2.2), collagen Iα1 (5.6 ± 2.0 versus 23.8 ± 7.3), and collagen III (2.7 ± 0.9 versus 7.6 ± 2.1). Data were corrected for rpl32 mRNA expression and expressed relative to Wistar Kyoto (WKY) rats [=1.0]. Expression of fibronectin protein was also lowered by eprosartan (0.8 ± 0.1 versus 1.9 ± 0.5), relative to WKY rats. Eprosartan provided significant renoprotection to SHR-SP rats as measured by decreased proteinuria (22 ± 2 versus 127 ± 13 mg/day) and histological evidence of active renal damage (5 ± 2 versus 195 ± 6) and renal fibrosis (5.9 ± 0.7 versus 16.4 ± 1.9) in vehicle- versus eprosartan-treated rats, respectively. Our results demonstrated that AT₁ receptor blockade with eprosartan can reduce blood pressure and preserve renal structure and function in this model of severe, chronic hypertension. These effects were accompanied by a decreased renal expression of transforming growth factor-β1, plasminogen activator inhibitor-1, and several other extracellular matrix proteins compared with vehicle-treated SHR-SP.

The renin-angiotensin system is a major regulator of blood pressure within the body, through the maintenance of vascular tone and sodium homeostasis. The renin-angiotensin system has, however, also been implicated in a number of diseases, characterized by remodeling and fibrosis, including forms of progressive renal disease. The generation of angiotensin II can lead to organ damage through both mitogenic activity and pro-fibrotic remodeling. Eprosartan is a potent (Kᵢ = 1.4 nM) angiotensin II receptor antagonist selective for the AT₁ subtype. AT₁ receptor antagonists have been shown to attenuate the effects of exogenous angiotensin II (Wang et al., 1997) and to be renoprotective in the partial nephrectomy model of renal failure (Gandhi et al., 1999), as measured by its ability to attenuate the hypertension, proteinuria, and up-regulation in the expression of several profibrotic genes associated with this model (Wong et al., 2000). TGF-β gene expression has been shown to be up-regulated in a number of animal models of fibrotic disease, including renal disease (Border and Noble, 1998) and can be induced by several different vasoactive mitogens, including angiotensin II (Klahr and Morrissey, 2000). This profibrotic cytokine mediates the up-regulation of several extracellular matrix component genes, including fibronectin and collagen, leading to increased synthesis of the extracellular matrix (Ignatzi and Massague, 1986). Furthermore, TGF-β can induce the expression of plasminogen activator inhibitor-1 (PAI-1), which inhibits the conversion of plasminogen to the active plasmin. Plasmin, in addition to lysing fibrin, can activate collagenases, which degrade collagen (Baricos et al., 1995), and has matrix-disintegrating effects. Thus, the inhibition of plasmin activation by PAI-1 prevents the breakdown of both thrombi and extracellular matrix, contributing to the increase in renal injury and scarring. In the present study, we have evaluated the effect of eprosartan in a model of severe chronic hypertension using spontaneously hypertensive stroke-prone rats (SHR-SP) fed a high-fat, high-salt diet.

Materials and Methods

Experimental Design. Male SHR-SP rats, progeny from the strain developed by Okamoto et al. (Yamori, 1974; Okamoto et al., 1997), were used in this study. Male SHR-SP rats, progeny from the strain developed by Okamoto et al. (Yamori, 1974; Okamoto et al., 1997), were used in this study.

Abbreviations: AT₁, angiotensin type 1 receptor; HFD, high fat/salt diet; PAI-1, plasminogen activator inhibitor-1; SHR-SP, spontaneously hypertensive rat-stroke prone; TGF-β, transforming growth factor β; WKY, Wistar Kyoto.
were obtained from the National Institutes of Health (Bethesda, MD) and were bred in the Department of Laboratory Animal Science at GlaxoSmithKline Pharmaceuticals (King of Prussia, PA). This investigation conformed with the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health (National Institutes of Health Publication 85–23, revised 1996). Procedures were approved by the Institutional Animal Care and Use Committee. Male SHR-SP rats between 10 and 13 weeks of age were adapted to individual cages and fed a powdered NIH-07 diet for 2 weeks before treatment assignment. On the basis of body weight and age, SHR-SP were assigned to three groups (n = 25 rats/group). Two of the groups received a diet of 1% NaCl as drinking water and chow supplemented with 24.5% fat (high fat/salt diet; HFD) and received intraperitoneal implants of Alzet osmotic mini-pumps (model 2 ML4; Alza Corp., Palo Alto, CA). Pumps were replaced every 28 days throughout the 12-week study under aseptic surgical conditions. Pumps contained either eprosartan (delivered at 60 mg/kg/day) or vehicle. The daily eprosartan dose was selected based on previous data in the rat demonstrating its renoprotective effects (Wong et al., 2000). The remaining SHR-SP group received normal water and chow (4.5% fat, NIH-07, 0.33% sodium, and 0.80% potassium) (SHR-SP normal diet group). WKY rats of the same age and weight were fed the normal diet and were also studied for control purposes (WKY normal diet group). To avoid unnecessary suffering, moribund animals were euthanized with a pentobarbital overdose, at that time an animal from each of the other treatment groups was randomly selected and tissues were harvested from these and the moribund rat as described in the histopathology section below. Eprosartan was synthesized at GlaxoSmithKline Pharmaceuticals (King of Prussia, PA). Diets were milled and formulated by Zeigler Brothers, Inc. (Gardners, PA). All feed and drinking solutions were provided ad libitum.

Body Weight, Blood Pressure, and Heart Rate. Body weight, systolic blood pressure, and heart rates were measured at 3-week intervals throughout the study. The systolic blood pressure and heart rate were measured using an automated tail-cuff method (model 179, IITC Life Science, Woodland Hills, CA).

Renal Function Determinants. Rats were placed in metabolism cages and 24-h urine samples were collected at weeks 5 to 7. Following collection, urine was stored at −20°C prior to assay of urinary protein excretion. This was determined using the sulfosalicylic acid method as previously described (Brooks et al., 1993), and 24-h excretion was calculated.

Histopathology. Tissues from a sample of eight to nine rats from each of the experimental groups were prepared for morphological examination after 6 to 11 weeks of the experiment. Whole body perfusion with 200 ml of phosphate-buffered saline (pH 7.2) followed by 300 ml of Bouin’s acetic acid solution (pH 1.8) was conducted immediately upon euthanasia using an overdose of pentobarbital. Kidneys were removed, weighed, stored in Bouin’s acetic acid for 16 h, and then transferred to 70% ethanol. The fixed organs were trimmed of excess adipose tissue. Standard central coronal transverse sections of each kidney were then processed for quantitative/semiquantitative histopathological evaluations. After dehydration and processing into paraffin, sections were cut at 6 μm and stained using Hematoxylin and Eosin (H&E) as described in detail previously (Luna, 1968). A multiparametered histopathological evaluation of kidney tissue was then performed.Crudex scores of renal damage were determined as described in detail previously (Volpe et al., 1990; Camargo et al., 1993; Barone et al., 1996; Wong et al., 1996). In brief, standard transverse sections were graded based on overall renal damage as follows: 0 (no damage), 0.5 (rare early arterial or arteriolar necrosis and/or mild glomerular and tubular-interstitial changes), 1.0 to 1.75 (necrosis/thrombosis of a few arterioles and/or arterioles with focal ischemia, thrombosis or hypercellularity of glomeruli, secondary tubular necrosis, and regeneration involving up to one-quarter of cortical and medullary parenchymal structures), 2.0 (moderate arterial necrosis/thrombosis with ischemic, thrombotic, and regenerative/hyperplastic changes involving up to one-half of the cortical and medullary parenchymal structures) or 2.5 to 3 (extensive necrosis/thrombosis of arteries with focal ischemic changes including infarcts of glomeruli and ischemic, thrombotic, and hypercellular/regenerative/ reparative changes in over one-half of the cortical and tubular parenchyma). Total active renal damage is the sum of the components, arterial necrosis/thrombosis, arterial proliferation, total glomerular lesions and foci of tubular regeneration. Total cortical and medullary casts are taken as an expression of irreversible nephron loss (chronic change). Renal fibrosis occurs as components of normal renal structures and as foci of pathologic fibrosis, i.e., scarring. Normal renal fibrosis is comprised primarily of adventitial fibrous tissue surrounding (interlobular and arcuate) arteries and occasionally of ligamentous-like bundles of fibrous tissue scattered in the parenchyma. Foci of interstitial nephritis and renal fibrosis are other expressions of chronic renal damage. All histological determinations were made in a completely blind manner. As much as possible, minor adjustments for ties in kidney crude scores were made before decoding (i.e., sections were coded and the analyses of each section was completed without any knowledge of treatment group classification).

Gene Expression. Total RNA was prepared from frozen tissues by guanidine thiocyanate denaturation. The expression of transforming growth factor β1 (TGF-β1), PAI-1, fibronectin, collagen I-α1, and collagen III was evaluated by quantitative RT-PCR using the Taqman Realtime 7700 system (Applied Biosystems, Foster City, CA). cDNA was synthesized from 2 μg of total RNA and diluted 20-fold. A
25-μl reaction volume containing 200 nM primers, 200 nM probe, and Master Mix (Applied Biosystems) was mixed with 2 μl of diluted cDNA and amplified by PCR. The thermal cycle conditions consisted of initial incubation steps of 30°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primer sequences for rat Tgf-β were as follows: probe, FAM-TGGTGGAC-CCGCAACAACGCCA-TAMRA; forward, AGAAGTCCACCCGGTG-CTGTA; and reverse, TGTGTAATGTGGTTTG GTA. The primer sequences for rat PAI-1 were as follows: probe, FAM-TTGTGCGT-A; and reverse, TGTGTGATGTCTTTGGTTTTGTCA. The primer sequences for rat fibronectin were as follows: forward, CAGCAGTGTTATGTAA. The primer sequences for rat collagen I were as follows: probe, FAM-TGGTGGAC-CCGCAACAACGCCA-TAMRA; forward, AGAAGTCCACCCGGTG-CTGTA; and reverse, TGTGTAATGTGGTTTG GTA.

The primer sequences for rat collagen I were as follows: probe, FAM-TGGTGGAC-CCGCAACAACGCCA-TAMRA; forward, AGAAGTCCACCCGGTG-CTGTA; and reverse, TGTGTAATGTGGTTTG GTA. The primer sequences for rat collagen I were as follows: probe, FAM-TGGTGGAC-CCGCAACAACGCCA-TAMRA; forward, AGAAGTCCACCCGGTG-CTGTA; and reverse, TGTGTAATGTGGTTTG GTA.

**Results**

**Survival.** SHR-SP rats fed a HFD demonstrated 95% mortality by 9 weeks on the diet. No mortality was noted in SHR-SP fed HFD treated with eprosartan or SHR-SP and WKY fed normal diet over the 12-week time period of the experiment (P < 0.001).

**Body Weight, Blood Pressure, Heart Rate, Urinary Protein Excretion, and Kidney Weight Indices.** SHR-SP rats demonstrated a significantly higher systolic blood pressure (SBP) at week 6 compared with normotensive WKY rats (Fig. 1). Feeding SHR-SP rats a HFD increased SBP further, however, this was significantly reduced by the administration of eprosartan. Heart rate was similar in all four groups (data not shown). The urinary protein excretion was significantly higher in the SHR-SP group fed a high-fat diet, but the rise in proteinuria was reduced to control levels by eprosartan administration (Fig. 1). The body weights of SHR-SP HFD rats at 6 weeks were significantly lower than the SHR-SP normal diet controls, but this decrease was normalized by eprosartan (Table 1). Eprosartan also normalized kidney weight indices.

**Gene and Protein Expression.** The renal expression of TGF-β1 mRNA was significantly higher in SHR-SP rats compared with WKY rats (Fig. 2). The expression was further increased by the HFD. Eprosartan attenuated the rise in TGF-β1 gene expression to a level that reached statistical significance (P < 0.05). PAI-1 mRNA levels were statistically higher in SHR-SP rats as compared with WKY rats (Fig. 2). Rats fed on a high-fat, high-salt diet showed a further increase in dry weight, which was abolished by the presence of eprosartan to a level close to that observed in normal diet SHR-SP rats. Fibronectin mRNA levels were significantly elevated in SHR-SP rats as compared with WKY rats. The high-fat, high-salt diet resulted in a further elevation, which was reduced by eprosartan (Fig. 3). The fibronectin protein expression paralleled mRNA levels (Fig. 3). Collagen I-α1 and collagen III gene expression displayed a similar pattern to that of TGF-β1 with the statistically significant increase of expression in SHR-SP HFD rats being attenuated by the administration of eprosartan (Fig. 4). TGF-β expression paralleled closely kidney crude score. Expressions of fibronectin protein and mRNA for TGF-β1, PAI-1, fibronectin,

**TABLE 1**

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<thead>
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<th>Effect of eprosartan (EPRO, 60 mg/kg/day) on body weight and kidney weight 6 to 11 weeks following feeding of a HFD</th>
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<td><strong>Normal Diet</strong></td>
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<td><strong>WKY</strong></td>
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<td><strong>Body weight (g) 6 weeks</strong></td>
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<td><strong>Kidney weight (g) terminal (6–11 weeks)</strong></td>
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<td><strong>Kidney weight index (g/100 g terminal BW)</strong></td>
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*P < 0.05 versus SHR-SP normal diet.
†P < 0.05 versus SHR-SP HFD.
collagen I, and collagen III followed patterns similar to histologically determined total renal fibrosis.

**Renal Histopathology.** Histopathologic scores of renal damage, as kidney crude scores, total active renal damage, and total renal casts (an approximation of chronic/irreversible renal damage) reveal that treatment with eprosartan reduces damage below that of both SHR-SP fed HFD and SHR-SP fed normal diet into the range of WKY normal diet rats. Examples of these lesions are illustrated in Figs. 5 to 8 and quantified in Table 2. Importantly, eprosartan reduced proliferative arterial lesions by 97% and necrotic/thrombotic arterial lesions by over 99%. Glomerular lesions, most (72%)...
well was equalized using the mRNA levels of ribosomal protein L32 (bottom) mRNA levels in SHR-SP rats fed a HFD. Amount of RNA per well was equalized using the mRNA levels of ribosomal protein L32 (rPL32). n = 6 per group; * P < 0.05 versus SHR-SP normal diet; † P < 0.05 versus SHR-SP HFD.

of which showed some degree of ischemic collapse were reduced by 98%; fibrous obliteration of glomerular tufts was rarely encountered. Eprosartan prevented pathological fibrosis.

Discussion

The administration of eprosartan, an AT1 receptor antagonist, to SHR-SP rats fed a high-fat, high-salt diet resulted in decreased hypertension and provided renoprotection as evidenced by protection of kidney structural integrity and a reduction in urinary protein excretion. These findings provide further support to the involvement of angiotensin II in the development of hypertension and renal damage, and indicate that AT1 receptor blockade is effective in a model of severe hypertension. Control of renal damage, as evidenced by kidney crude score, was comparable with that achieved with losartan in SHR-SP (Camargo et al., 1993), but pressure control is difficult to compare due to differences in the dietary management and drug administration in the two studies.

The progression of renal failure is a process involving several examples of autoinduction and positive feedback loops. Because morphological studies of arterial alterations in early experimental hypertension feature karyorhexis (Campbell and Santos-Buch, 1966) and increased nuclear uptake of tritiated thymidine (Crane and Dutta, 1963), mitogenic activity may initiate vascular necrosis, which subsequently results in thrombosis and ischemic damage to nephrons. Additional changes may result from the uncontrolled actions of normal repair mechanisms that are locked in a vicious cycle of activation due to repeated and/or constant injury. The hypertension observed in the present study was due to an activated renin-angiotensin system as evidenced by the reduction in systolic blood pressure with the administration of eprosartan. Angiotensin II has been shown to possess both hemodynamic and nonhemodynamic effects (Johnson et al., 1992; Kagami et al., 1994), although it is difficult to assess their relative contributions toward the progression of renal failure because they are intrinsically linked. Angiotensin II can cause an increase in the glomerular capillary hydraulic pressure (P_g) secondary to the glomerular hypertension (Myers et al., 1976; Blantz et al., 1976; Anderson et al., 1985). This leads to the development of proteinuria and increased shear stress, resulting in the local release of cytokines.

Independent of its effects on blood pressure, angiotensin II may directly stimulate the up-regulation of cytokine expression, most notably TGF-β1 (Klahr and Morrissey, 1998). This cytokine is the most characterized mediator of extracellular matrix production, and it has been shown to stimulate the synthesis of a number of matrix components and their receptors (Border and Noble, 1994). It has also been shown to induce an increase in PAI-1, which is important in preventing breakdown of thrombi and interstitial matrix. In our study, the administration of eprosartan resulted in a decrease in necrotic arterial disease, thrombosis and expression of TGF-β1 and a number matrix components.

Our observation that blockade of the renin-angiotensin system with an AT1 receptor antagonist can attenuate the enhanced TGF-β1, PAI-1, and matrix protein expression in renal disease is consistent with previous reports in a number of models of renal disease including partial nephrectomy (Junaid et al., 1997; Wu et al., 1997; Ali et al., 1998; Noda et al., 1999; Taal et al., 2001), immune-mediated renal injury (Yayama et al., 1995; Hisada et al., 1999; Taal et al., 2001), hypertensive glomerulosclerosis (Nakamura et al., 1997; Zoa et al., 1998; Peters et al., 2000), hypertension-induced renal disease (Obata et al., 1997; Otsuka et al., 1998; Wolf et al., 1998), unilateral ureteral obstruction (Ishidoya et al., 1995), cyclosporine nephrotoxicity (Shihab et al., 1997), and the diabetic transgenic (mRen-2)27 rat (Kelly et al., 2000). Angiotensin II has been shown previously to stimulate TGF-β1 expression directly in renal cells (Klahr and Morrissey, 1998). However, angiotensin II may also increase TGF-β1 indirectly by enhancing the development of proteinuria. Thus, increased proteinuria, itself, has been demonstrated to induce TGF-β1 expression (Remuzzi et al., 1997) and increased protein excretion can lead to tubulointerstitial fibrosis and further progression of renal disease. Certainly, the hemodynamic effects of angiotensin II blockade in the glomerulus, e.g., reduced glomerular hypertension, would be expected to reduce the proteinuria (Kagami et al., 1994).

The mechanism of increased PAI-1 expression observed in the present study may involve a number of different factors because both TGF-β1 (Zelenza et al., 1992; Okuda et al., 1990) and angiotensin II (Gesualdo et al., 1999) can induce...
PAI-1. In addition, it has been suggested that the angiotensin II metabolite, angiotensin IV, rather than angiotensin II itself can lead to an increase in PAI-1 expression (Kerins et al., 1995). Because eprosartan has no affinity for the AT₄ receptor (R. M. Edwards, unpublished observations), our data would suggest that, either directly or indirectly via TGF-β, angiotensin II, and not angiotensin IV, induction is involved in increasing PAI-1 expression under the present conditions.

It is unclear from the present study whether reduction in expression TGF-β, PAI-1, and matrix proteins was due to the lowering in blood pressure induced by eprosartan or inhibition of the profibrotic effects of angiotensin II. If the effects of angiotensin II on these components are indeed receptor-mediated, then it is unlikely that they can be separated from the blood pressure lowering effects of eprosartan. It should be noted, however, that carvedilol can reduce renal damage in the spontaneously hypertensive stroke-prone rat without lowering blood pressure (Barone et al., 1996) indicating that a decrease in blood pressure is not necessary for renal protection.

In summary, our study showed that in a model of severe chronic hypertension the AT₁ receptor antagonist, eprosartan, was able to reduce systolic blood pressure and attenuate dramatically the progression of renal disease. Furthermore, eprosartan reduced the expression of TGF-β1, PAI-1, and the matrix proteins fibronectin and collagens I-α1 and III.
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References


Fig. 7. Afferent arterioles (indicated by single arrows) and glomeruli. A, a WKY rat fed a normal diet with normal arteriolar (single arrow), glomerular, and tubular structure. B, a SHR-SP fed a normal diet with a glomerulus and fibrinoid necrosis of its afferent arteriole (single arrow) and its hilar area. Double arrows indicate a glomerulus with ischemic collapse and proliferative glomerulopathy. C, a SHR-SP fed HFD with ischemic collapse of a glomerular tuft (double arrows), sclerosis/fibrosis of its hilar arteriole (single arrow) and regeneration of adjacent tubules. TC indicates tubular cast. D, a SHR-SP fed HFD and treated with eprosartan showing normal arteriolar (single arrow), glomerular, and tubular structure. Bar = 27 μm.

Fig. 8. Low power illustrations of midcortical regions to demonstrate extent of tubulointerstitial damage. A, a WKY rat fed a normal diet with normal glomeruli and tubulointerstitial structures. B, a SHR-SP fed a normal diet with a few tubular casts (TC) in lower, right corner but otherwise unremarkable structure. C, a SHR-SP fed HFD with extensive tubular regeneration (dilated tubules with low/cuboidal epithelial linings), scattered tubular casts (TC), and early periglomerular and peritubular fibrosis near centrally located large glomerulus. Arrow indicates glomerulus with ischemic collapse. D, a SHR-SP fed HFD and treated with eprosartan showing normal glomeruli and tubulointerstitial structures. Bar = 67 μm.


