Original Article

Role of Aberrant Iron Homeostasis in the Upregulation of Transforming Growth Factor- β 1 in the Kidney of Angiotensin II-Induced Hypertensive Rats

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We have previously shown that abnormal iron metabolism might be one underlying mechanism of the renal damage observed in the angiotensin II-infused rat. Transforming growth factor- β 1 (TGF- β 1) is known to play a crucial role in the development of renal damage induced by activation of the renin-angiotensin-aldosterone system. The purpose of the present study was to examine the effects of an iron chelator and a free radical scavenger on the angiotensin II-induced upregulation of TGF- β 1 in the kidney. Rats were given angiotensin II (0.7 mg/kg/day) *via* osmotic minipumps for 7 days. The expressions of the mRNAs of TGF- β 1 and collagen types I and IV were significantly increased in response to angiotensin II treatment. Histologic analysis showed that TGF- β 1 expression was upregulated mainly in tubular epithelial cells, and occasionally in glomerular and perivascular cells, some of which were identified as monocytes and/or macrophages. Although tubular cells that overexpressed TGF- β 1 did not contain iron particles, angiotensin II-induced TGF- β 1 upregulation was suppressed by the iron chelator and the free radical scavenger. The free radical scavenger also suppressed angiotensin II-induced upregulation of heme oxygenase-1, an oxidative-stress sensitive gene. By contrast, administration of iron dextran to rats induced upregulation of TGF- β 1 mRNA. Collectively, these data suggest that the renal iron overload and presumed subsequent increase in oxidative stress play a role in angiotensin II-induced upregulation of the mRNAs of TGF- β 1 and collagen types I and IV in the kidney. (*Hypertens Res* 2004; 27: 599–607)

Key Words: angiotensin II, fibrosis, iron chelator, radical scavenger, kidney

Introduction

Transforming growth factor- β (TGF- β) is postulated to be one of the key molecules involved in the pathogenesis of progressive glomerular and tubulointerstitial scarring and renal failure human kidney disease and various experimental models thereof (1). It has been shown that blockade of the angiotensin type 1 (AT₁) receptor signaling pathway de-

creases the renal expression of TGF- β in animal models of glomerulosclerosis (2, 3), hypertension (4), and diabetes (5), and this decrease is accompanied by a decrease in urinary excretion of protein. In addition, administration of exogenous angiotensin II to rats has been shown to induce renal expression of TGF- β 1 (6). The idea that TGF- β plays a crucial role in the development of renal damage was supported by the recent findings that anti-TGF- β antibody ameliorates renal damage in some animal models (7, 8); therefore, induc-

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tion of TGF- β may be involved in a mechanism of renal injury evoked by activation of the renin-angiotensin-aldosterone system.

We recently reported that long-term administration of angiotensin II causes iron deposition in the renal tubules (9). Iron is known to produce highly toxic hydroxyl radicals from less toxic hydrogen peroxide through the Fenton reaction. Thus iron may further enhance the oxidant-induced renal damage initiated by angiotensin II, a peptide which is known to activate NAD(P)H oxidase and overproduce superoxide in the kidney (10). We also found that iron chelation suppressed the increase in proteinuria (9) and downregulation of the *klotho* gene, an anti-aging gene which may play a renoprotective role, in angiotensin II-treated rats (11, 12). These findings indicate that angiotensin II-induced renal iron deposition is a phenomenon that may have potential for modulating renal function and gene regulation.

Possible linkage between iron overload and induction of TGF- β 1 and other pro-fibrotic genes has been suggested in hepatic (13, 14) and cardiac cells (15). In addition, recent studies have reported that renal TGF- β gene expression is upregulated in response to iron-mediated oxidative stress, resulting in tubulointerstitial damage (16). These findings lead us to hypothesize that there may be a link between iron overload and angiotensin II-induced upregulation of TGF- β in the kidney. In this study, we investigated whether iron chelation and a free radical scavenger suppressed angiotensin II-induced upregulation of TGF- β and collagen genes.

Methods

Generation of the Animal Models

Angiotensin II was continuously infused into male Sprague-Dawley rats by subcutaneous implantation of an osmotic minipump (Alzet model 2001; Alza Pharmaceutical, Mountain View, USA) as described previously (17). Briefly, Val5-angiotensin II (Sigma, St. Louis, USA) was infused at a dose of 0.7 mg/kg/day for 7 days. In some experiments, rats were given daily subcutaneous injections of an iron chelator, deferoxamine (a gift from Novartis), at a dose of 200 mg/kg/day. Iron-overload was induced by an intraperitoneal injection of an iron-dextran complex (a gift from Teikoku Hormone Mfg.) at a dose of 240 mg of equivalent of elemental iron/kg on Days 0, 2, 4, and 6 of angiotensin II infusion. In some experiments, a new free radical scavenger, T-0970 (18) (a gift from Tanabe Seiyaku), was given orally at a dose of 10 mg/kg/day. The effect of T-0970 as a radical scavenger in our system has been demonstrated elsewhere (12). Systolic blood pressure (SBP) was measured in conscious rats by tail-cuff plethysmography (Ueda Seisakusyo, Tokyo, Japan).

cDNA Probes and Northern Blot Analysis

Rat TGF- β 1 cDNA probe was a gift from Shioh-Shih Tang (Harvard Medical School, Massachusetts General Hospital, Boston, USA) and John S.D. Chan (Centre Hospitalier de l'Université de Montréal-Hôtel-Dieu, Montreal, Canada). Rat collagen type I and IV cDNAs were obtained by subcloning the reverse-transcription-polymerase chain reaction (RT-PCR) product using rat kidney mRNA. The sense and antisense primers were 5'-TGCCGTGACCTCAAGATGTG-3' and 5'-CACAAGCGTGCTGTAGGTGA-3', respectively, for collagen type I, and 5'-GCGAAGCGCCCGCCATGGTGAT-3' and 5'-TGACGGCGGCAGAGGGGGGCAT-3', respectively, for collagen type IV. RT-PCR products were subsequently subcloned into the plasmid vector using the pGEM-T vector system (Promega, Madison, USA) and validated by DNA sequencing using the dideoxyribonucleotide sequencing method.

Total RNA was obtained using Isogen (WAKO, Osaka, Japan), and mRNA was subsequently isolated using oligotex-dT30 (Roche Diagnostics, Indianapolis, USA). cDNA probes encoding TGF- β 1 or collagen genes were labeled with [α - 32 P]dCTP (Amersham Life Sciences, Piscataway, USA) using commercially available kits (Nippon Gene, Tokyo, Japan). Hybridized bands were visualized, and quantified using a bio-imaging analyzer (BAS 2000; Fuji Photo Film, Tokyo, Japan), and band densities were normalized to the intensities of corresponding GAPDH mRNA bands of the samples on the same gel.

In Situ Hybridization, Histologic and Immunohistochemical Analyses

Rat TGF- β 1 cDNA was subcloned into pGEM-T vector in sense and antisense orientations by standard methodology. After digestion with a restriction enzyme and linearization of the plasmid, antisense and sense cRNA riboprobes were transcribed *in vitro* using the DIG RNA labeling Kit SP6/T7 (Roche Diagnostics, Mannheim, Germany). Hybridization was performed using In Situ Hybridization Reagents (Nippon Gene) according to the manufacturer's instructions. Prussian blue staining was performed to detect tissue iron deposition. In some specimens, immunohistochemistry was performed after *in situ* hybridization using antibodies against monocytes/macrophages (ED-1; Chemicon, Temecula, USA) and human α -smooth muscle (α -SM) actin (Sigma) at a dilution of 1/100. Laser scanning confocal fluorescence microscopy combined with differential interference contrast imaging was performed using FLUOVIEW FV300 (Olympus, Tokyo, Japan).

Protein Purification and Western Blot Analysis

Protein was isolated by homogenizing samples in the lysis buffer (50 mmol/l HEPES, 5 mmol/l EDTA, and 50 mmol/l

Fig. 1. Time course of the expression of TGF- β 1 and extracellular matrix genes after administration of angiotensin II (ANG II). Rats were treated with ANG II at a dose of 0.7 mg/kg/day for up to 7 days. The left panel shows a representative Northern blot, and the right panel summarizes the data of 5–7 rats in each group. * $p < 0.05$ and † $p < 0.01$ vs. untreated controls.

NaCl; pH 7.5) containing protease inhibitors (10 μ g/ml aprotinin, 1 mmol/l PMSF, and 10 μ g/ml leupeptin). Equal amounts of protein were loaded onto 15% SDS polyacrylamide gels and subsequently blotted onto Immobilon-P polyvinylidene difluoride membranes (Millipore, New Bedford, USA). Polyclonal antibodies against rat heme oxygenase 1 (HO-1) (SPA895) (StressGen, Victoria, Canada) and rat ferritin (Panapharm, Kumamoto, Japan) were used at a 1/2,000 dilution, and horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, USA) was used at a 1/2,000 dilution. The ECL Western blotting system (Amersham Life Sciences, Arlington Heights, USA) was used for detection. Bands were visualized using a lumino-analyzer (LAS-1000; Fuji Photo Film). Band intensity was calculated using NIH image analysis software (NIH, Research Service Branch, Bethesda, USA), and was expressed as a percentage of the control value.

Oxidative Fluorescent Microscopy

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate *in situ* production of reactive oxygen species. Unfixed frozen sections of 30- μ m thickness were incubated with 2 μ mol/l DHE, with or without co-incubation of superoxide dismutase-polyethylene glycol (PEG-SOD; 500 U/ml), at 37°C for 30 min. Images were obtained with a laser scanning confocal microscope, FLUOVIEW FV300.

Statistical Analysis

Data were expressed as the mean $\pm$ SEM. Results were presented as a percentage of the control value. A value of $p < 0.05$ was considered to be statistically significant.

Results

Regulation of mRNA Levels of TGF- β 1 and Collagen Genes

The SBP of control rats was 131 ± 3 mmHg, and it was elevated at day 1 (153 ± 4 mmHg, $p < 0.05$ vs. control), day 3 (174 ± 3 mmHg, $p < 0.01$ vs. control), day 5 (191 ± 3 mmHg, $p < 0.01$ vs. control), and day 7 (192 ± 4 mmHg, $p < 0.01$ vs. control) after angiotensin II infusion. Administration of angiotensin II significantly increased the mRNA expressions of TGF- β 1 and collagen types I and IV as early as 1 day after infusion (Fig. 1).

Histologic and Immunohistochemical Analyses

In situ hybridization showed that TGF- β 1 was weakly expressed in the tubular epithelial and mesangial cells in the kidney of control rats (Fig. 2A). TGF- β 1 mRNA expression in these areas was markedly enhanced after angiotensin II infusion (Fig. 2B, C). Prussian blue staining of the serial sections showed marked iron deposition (blue colors) in the tubular epithelial cells (Fig. 2D); however, tubular cells with high TGF- β 1 mRNA expression (asterisks in Fig. 2B, D) did not contain apparent iron particles. Higher magnification showed that angiotensin II also increased the expression of TGF- β 1 mRNA in the mesangial cells (Fig. 2E–H). Next, we stained the specimens with ED-1, an antibody to identify monocytes/macrophages, and α -SM actin after *in situ* hybridization. ED-1-positive cells were observed in the interstitium as well as in the intraluminal space, and these cells occasionally expressed high levels of TGF- β 1 mRNA (Fig. 2I, J). Vascular smooth muscle cells did not express high levels of TGF- β 1 mRNA (Fig. 2K, L).

Fig. 3. Effect of iron chelation, iron overload, and free radical scavenging on the angiotensin II-induced upregulation of TGF- β 1 and collagen genes. The upper panel shows representative Northern blots, and the lower panel summarizes the data of 5–7 rats in each group. * $p < 0.05$ and † $p < 0.01$ vs. untreated controls. # $p < 0.05$ vs. angiotensin II-treated rats.

scavenger. T-0970 treatment suppressed the upregulation of both ferritin and HO-1 induced by angiotensin II (Fig. 5).

Superoxide Production in the Renal Cells with Iron Deposition

Finally, we investigated the superoxide production by fluorescent microscopy. Fluorescence intensity was apparently increased in some regions in the kidneys of angiotensin II-infused rats compared to those of control rats. Higher magnification revealed that cells that were positive for granular materials, as visualized by Nomarski differential interference contrast (DIC) imaging, showed the greatest increase in Ethidium Bromide (EtBr) staining, and thus in superoxide production (Fig. 6). However, these materials were not identified as histologically stainable iron. Conversely, tubular cells that were most strongly stained with EtBr were not cells with histologically stainable iron (Fig. 6G–I).

Discussion

In this study, we demonstrated that long-term administration of angiotensin II upregulated the renal expression of TGF- β 1 and collagen types I and IV, and this effect was suppressed by the treatment of the rats with an iron chelator or a free radical scavenger. Conversely, expression of these genes was induced in rats with iron overload induced by administration of iron dextran. These findings suggest that the angiotensin II-induced production of TGF- β 1 and collagen genes in the kidney may be, at least in part, mediated by renal iron accumulation and, presumably, by a subsequent increase in the extent of oxidative stress.

The role of iron in the development of tissue fibrosis has been most extensively demonstrated in the liver. Iron overload is postulated to be one of the mechanisms that increase TGF- β expression and/or enhance hepatic fibrosis in alcoholic liver disease (19), chronic hepatitis C virus infection

Fig. 4. Azan staining. A, B: Control rats. C, D: Rats given angiotensin II for 7 days. Modestly increased perivascular fibrosis could occasionally be observed in the kidneys of angiotensin II-infused rats. Original magnifications: $\times 250$ (A, C), $\times 800$ (B, D).

(20), and genetic hemochromatosis (14). The findings that the amount of oxidized lipid was increased in the liver of animal models of iron overload and that antioxidant treatment reduced the development of fibrosis in animals with iron overload (21) support the notion that increased production of reactive oxygen species (ROS) mediates fibrotic properties of iron overload. Although the possible link between iron overload and renal damage has been less extensively studied in the past, several recent studies have suggested a possible association between iron-mediated oxidative stress and renal damage in the kidney. Zhou *et al.* have reported that administration of iron dextran to rats increased oxidized lipid, nitrotyrosine, proteinuria, glomerulosclerosis, tubular atrophy, and interstitial fibrosis, and these findings were diminished in the animals with iron depletion (22). Nath *et al.* reported that heme protein overload, induced by the injection of glycerol, decreased glomerular filtration rate and increased the expression of TGF- $\beta 1$ and collagen mRNAs (16). These findings suggest that excessive iron may induce renal injury and upregulate TGF- $\beta 1$ expression by increasing the extent of oxidative stress, presumably *via* catalysis of the Fenton reaction to generate toxic hydroxyl radicals. We previously found that long-term administration of angiotensin II causes iron deposition in the renal cells and increased proteinuria (9). Thus, the data of the present study may suggest the possibility that alteration of iron homeostasis could potentially modulate the expression of several genes, such as pro-fibrotic genes and anti-aging genes (12), in the kidney of angiotensin II-infused rats. In the present study, however, administration of iron dextran also slightly, though not signifi-

Fig. 5. Effect of iron chelation on the angiotensin II-induced upregulation of ferritin and heme oxygenase-1 (HO-1) proteins. The upper panel shows representative Western blots, and the lower panel summarizes the data of 4–6 rats in each group. * $p < 0.05$ and $^{\dagger} p < 0.01$ vs. untreated controls. $^{\#} p < 0.05$ vs. angiotensin II-treated rats.

cantly, increased blood pressure; therefore, the synergistic effects of hemodynamic stress and iron-mediated stress on TGF- $\beta 1$ expression in the kidney should be investigated in future studies.

We showed that upregulation of TGF- $\beta 1$ mRNA after angiotensin II treatment occurred mainly in tubular epithelial cells, and occasionally in glomerular cells and migrated inflammatory cells. Tubular cells have also been reported to be the main contributors to renal TGF- $\beta 1$ induction in several other animal models, such as models of diabetic nephropathy (23) and obstructive nephropathy (24). Although the mechanisms underlying TGF- $\beta 1$ upregulation in tubular cells are not yet fully understood, ROS are thought to act as intracellular messengers to induce renal TGF- $\beta 1$ expression in some animal models (25, 26). In the present study, angiotensin II-induced TGF- $\beta 1$ upregulation was inhibited by deferoxamine, an iron chelator, and T-0970, a free radical scavenger. In addition, angiotensin II-induced upregulation of the renal expression of HO-1, an oxidative stress-sensitive gene, was inhibited by deferoxamine (9) and T-0970 (Fig. 5). We previously found that the increased production of ROS induced by angiotensin II could be inhibited by treatment with deferoxamine and T-0970, and mimicked by iron overload (12).

Fig. 6. In situ detection of superoxide in rat kidney. Fluorescent images of confocal microscopy of kidneys labeled with oxidative dye dihydroethidium (DHE). A: Kidney of a control rat. B–I: Kidney of an angiotensin II-infused rat. D–F are the same section. G and H are the same section, and I is a serial section of G (= H), corresponding to the bracketed region in H. Compared to that in the control (A), the fluorescence intensity was increased in the angiotensin II-infused rat kidney (B). When a section from an angiotensin II-infused rat was incubated with PEG-SOD at the time of DHE treatment, EtBr signal fluorescence was almost abolished (C). Higher magnification showed that EtBr fluorescence was increased (D) in the tubular cells that contained granular materials as demonstrated by Nomarski differential interference contrast imaging (E). F is a merged image of D and E. Although tubular epithelial cells with iron deposition seemed to have increased EtBr fluorescence compared to some other tubular cells (arrows in G–I), the serial sections showed that the granular materials were not histologically stainable iron and that cells that were most strongly stained with EtBr were not cells with histologically stainable iron (arrowheads in G–I). Scale = 20 μ m.

These data suggest the possibility that increased oxidative stress, which may be augmented by abnormal iron homeostasis, may be a cause of renal TGF- β 1 induction in the rat model of angiotensin II infusion. In the liver of patients with genetic hemochromatosis, TGF- β 1 was induced in the infiltrating cells with iron accumulation (14). In the present study, on the other hand, tubular cells that expressed high levels of TGF- β 1 mRNA were not identical with those showing iron deposition. We do not know the reason for this dissociation; however, there may be some factors released from iron-positive cells that could act to induce TGF- β 1 expression in neighboring cells in a paracrine manner. This possibility should be studied in future studies.

One may question the origin of deposited iron in the kid-

ney in angiotensin II-infused rats. We noticed several similarities between the heme-protein overload model (16) and the angiotensin II-infusion model (9); in both models, deposition of iron and upregulation of HO-1 could be observed in the tubular epithelial cells. These findings might suggest that administration of angiotensin II causes heme protein overload. In fact, degeneration of cardiac (27) and skeletal muscle (28) has been reported to occur in angiotensin-treated rats, and these degenerations may result in the release of myoglobin into the systemic circulation. It is thus possible that in the angiotensin II-infused rat model, re-absorption by tubular epithelial cells of the filtered iron derived from the heme-protein of degraded muscles may result in the overproduction of toxic free radicals *via* the Fenton reaction, a sce-

nario similar to that suggested to occur in rat remnant kidneys (29).

We showed that T-0970 inhibited angiotensin II-induced upregulation of renal ferritin protein. Iron staining also showed that T-0970 decreased the extent of iron accumulation in the kidney of angiotensin II-infused rats (data not shown). The mechanism of this phenomenon was not elucidated in the present study; however, it was possible that the free radical scavenger protected against angiotensin II-induced muscle damage (Ishizaka N, unpublished observation) and subsequently ameliorated the destruction of iron homeostasis in the kidney. It was also suggested that T-0970 may decrease renal oxidative stress not only by scavenging generated ROS, but also by decreasing the generation of ROS by inhibiting the Fenton reaction. In the last part of the present study, we showed that superoxide production was increased in the kidney of angiotensin II-infused rats by fluorescence microscopy. A previous report suggested that iron loading may increase the mitochondrial production of superoxide (30). Although superoxide production was greatest in the cells with granular materials as shown by DIC imaging, these granular materials were not identified as stainable iron (Fig. 6G–I). Therefore, the increase in the generation of superoxide was considered to be mild, if existent, in tubular epithelial cells with positive iron deposition.

In conclusion, long-term administration of angiotensin II caused upregulation of TGF- β 1 and collagen mRNAs. This phenomenon was blocked by an iron chelator and mimicked by iron overload. In addition, a free radical scavenger also blocked angiotensin II-induced upregulation of these fibrosis-related genes. These data suggest that aberrant iron homeostasis and subsequent increase in ROS production may be one underlying mechanism of the angiotensin II-induced upregulation of TGF- β 1 and collagen genes in the kidneys of angiotensin II-induced hypertensive rats.

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