Telmisartan inhibits both oxidative stress and renal fibrosis after unilateral ureteral obstruction in acatalasemic mice

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Abstract

Background. Reactive oxygen species are involved in many of the angiotensin II signalling pathways. We have thus investigated whether the angiotensin II type 1 (AT1) receptor antagonist, telmisartan, can inhibit the accelerated renal fibrosis and excess oxidative stress, which occurs after unilateral ureteral obstruction (UUO) in acatalasemic mice.

Methods. The effect of daily intraperitoneal injection of telmisartan (0.1–0.3 mg/kg body weight) on the renal tubulointerstitial injury induced by UUO has been studied in homozygous acatalasemic mutant mice (C3H/AnLCsbCsb) and wild-type mice (C3H/AnLCsaCsa). We evaluated the systemic blood pressure of the mice on the seventh day. In addition, the tubulointerstitial expression of collagens type I and type IV, the p22-, p47- and p67-phox subunits of NADPH oxidase, 4-hydroxy-2-nonenal, and 4-hydroxy-2-hexenal lipid peroxidation products were assessed by immunohistochemistry. The level of apoptosis was determined by terminal deoxynucleotidyl transferase nick end-labelling analysis, while the mRNA level of the p22-, p47- and p67-phox subunits was quantified by real-time PCR. The renal content of each of the antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase was determined by specific assay.

Results. Obstructed kidneys from acatalasemic mice exhibited increased tubulointerstitial deposition in dilated tubules of collagens type I and IV, lipid peroxidation products, and the p22/p47/p67-phox subunits of NADPH oxidase. The level of the p22/p47/p67-phox subunit mRNA, and of apoptosis in tubular epithelial cells, was also increased compared with those from wild-type kidneys. Treatment with telmisartan attenuated all of the changes and prevented renal fibrosis in a dose-dependent manner; despite the low dose (0.1 mg/kg). The treatment did not lower the systemic blood pressure. The catalase activity remained low in acatalasemic obstructed kidneys without compensatory upregulation of glutathione peroxidase or superoxide dismutase activity; the level of neither anti-oxidant enzymes in obstructed kidneys was affected by telmisartan.

Conclusions. The AT1 receptor antagonist telmisartan ameliorated renal fibrosis after UUO by inhibition of oxidative stress, even under acatalasemic conditions.

Keywords: acatalasemia; angiotensin II receptor antagonist; apoptosis; catalase; NADPH oxidase

Introduction

The renin–angiotensin system plays crucial roles in cardiovascular and renal pathophysiology. All components of the system are present in the kidney and constitute the local and functional renin–angiotensin system. Angiotensin II (Ang II) binds to its two major high-affinity receptors, designated AT1 and AT2. Signalling through the AT1 receptor results in vasoconstriction and sodium reabsorption, and also promotes cellular growth, hypertrophy, activation of fibroblasts, and extracellular matrix deposition in the kidney. Recently, randomized clinical trials have demonstrated that an AT1 receptor antagonist, losartan, has significant renoprotective effects on non-diabetic [1] as well as diabetic nephropathy. Ang II seems to play one of the central roles in the pathogenesis of renal interstitial fibrosis [2]. An analysis of AT1a gene knockout mice demonstrated the
inhibitory effect of AT1 blockade on renal interstitial fibrosis [3]. Several experimental studies suggest that the concentration of Ang II in the renal interstitium may be 60–100-fold higher than that in the circulation [4]. However, it is unknown how a further increase in Ang II in the renal interstitium is able to lead to fibrosis. Molecular and cellular damage occurring within the kidney would have to take place in response to increases in intrarenal Ang II above the already high basal level. Furthermore, the mechanism by which Ang II upregulation is sensed by the kidney is unknown.

Recent studies have shown that reactive oxygen species (ROS) are involved in many of the Ang II signalling pathways [5]. Ang II stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity via the AT1 receptor to produce the superoxide anion, hydrogen peroxide, and hydroxyl radicals, which are reported to exert effects on intracellular growth-related proteins and enzymes to mediate the final biological responses. ROS mediates Ang II stimulation of certain important signals, such as epidermal growth factor receptor transactivation, p38 mitogen activated protein kinase and Akt. Recently, a new family of NADPH oxidases has been discovered and shown to play an important role in cardiovascular diseases such as atherosclerosis and hypertension [6]. The subunits of this oxidase include a membrane-associated 22 kDa α-subunit (gp91phox) and a 91 kDa β-subunit (gp91phox), as well as the cytosolic components p47phox, p67phox and p40phox. Assembly of these subunits incorporates the small ATPases rac1 and rac2. The expression of NADPH oxidase and its subunits within the kidney should yield insight into Ang II-mediated ROS generation.

Unilateral ureteral obstruction (UUO) is a well-established experimental model for the study of the mechanisms of renal interstitial fibrosis and the evaluation of potential therapeutic approaches to ameliorate the fibrosis [2]. Vander [7] was the first to report the stimulation of renin release from the obstructed kidney after elevation of ureteral pressure by ureteral obstruction. UUO is associated with increases in Ang II concentration, and in angiotensin-converting enzyme activity, as well as in the renin content of the kidney. We previously reported that renal fibrosis was significantly reduced in AT1a receptor gene knockout mice after UUO [3]. The mutant mice displayed blunted TGF-β1 mRNA, and type III and IV collagen expression, as well as interstitial volume expansion, early in the course of the disease. Our recent study utilizing acatalasemic mice suggested that ROS were also important mediators in the progression of renal fibrosis in UUO [8]. A functional catalase deficiency sensitized tubular epithelial cells to apoptosis and accelerated oxidant tissue injury, leading to renal fibrosis in the mice. In these reports, however, the effect of AT1 receptor blockade on Ang II-mediated generation of ROS in renal fibrosis was not investigated. Since ROS are involved in many Ang II signalling pathways, we investigated whether the AT1 receptor antagonist, telmisartan, could inhibit oxidant-mediated tissue injury and renal fibrosis after UUO in acatalasemic mice.

Subjects and methods

Experimental protocol

Male wild-type mice (C3H/AnLcsCsds) and male homozygous acatalasemic mutant mice (C3H/AnLcsCsds) at 7–10 weeks of age were used in the experiments [8]. The development and fertility of the acatalasemic mice were normal. At the start of the experiments, the body weight (BW) was as follows: wild-type mice, 27.6 ± 1.02 g; acatalasemic mice, 26.7 ± 1.06 g. Mice were divided into subgroups (n = 8/group), and no mice died during the experimental period. UUO was performed as described previously [8]. Telmisartan (BIBR 277), an AT1 receptor-specific antagonist, was dissolved in phosphate buffered saline (PBS), adjusted to pH 8.0, and injected intraperitoneally 0–7 days after UUO at a dose of 0.1 or 0.3 mg/kg BW. A vehicle-treated group received the intraperitoneal injection of PBS alone. Sham operations were performed as controls; mice had their ureters manipulated but not ligated. The whole kidney weight was determined and expressed as a percentage of BW at the time the mice were sacrificed. Arterial blood pressure was measured using a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan) by the tail-cuff method. The Ethics Review Committees of Okayama University Graduate School of Medicine and Dentistry approved the experimental protocol for Animal Experimentation.

Reagents and antibodies

Chemicals and reagents of analytical grade were purchased from Sigma Co. Ltd (St Louis, MO, USA) or Wako Pure Chemical Ind. (Osaka, Japan) unless stated. The compound meso-Tetrakis (1-methyl-4-pyridyl)-porphyrin was purchased from Dojindo Labs (Kumamoto, Japan). The following primary antibodies were used for immunohistochemistry: mouse monoclonal antibodies to 4-hydroxy-2-nonenal (HNE) and 4-hydroxy-2-hexenal (HHE) were obtained from Chemicon International (Temecula, CA, USA); and antibodies against NADPH oxidase components p22phox, p47phox, p67phox and p40phox from Santa Cruz Biotechnology, Inc. (CA, USA).

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from the whole kidney by using an RNAsin Midi kit (Qiagen, Valencia, CA) as described previously [3]. Two micrograms of total RNA from each sample were used for reverse transcription (RT) using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA), Random hexamer (2.5 µM), MuLV reverse transcriptase (50 U) and deoxyribonucleoside triphosphate (1 mM) were used in the real time (RT) step at 42°C for 15 min, 99°C for 5 min and 5°C for 5 min. For the quantification of mRNA
levels, real-time PCR was performed using a LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostics, Mannheim, Germany). The oligonucleotide primers used for RT-PCR were custom-ordered from Nihon Gene Research Lab’s Inc. (Sendai, Miyagi, Japan). The primer sequences were as follows: mouse NADPH oxidase subunit p47-phox [9], forward 5’-ACCTGTCCGGAAGGTTGTT-3’, reverse 5’-TAGGCTCGAAGGTATGG-3’, cDNA size 381 bp, and GenBank accession no. AB002663; p22-phox, forward 5’-TGCGGGACGCTTCACGCAGTTG-3’, reverse 5’-GGTTGTTAGGTGCTGTATG-3’, cDNA size 376 bp, and GenBank accession no. U18729; p67-phox, forward 5’-AGGACTATCTGGGCAGGC-3’, reverse 5’-GCTGCGACTGAGGGTGAAAT-3’, cDNA size 332 bp, and GenBank accession no. AB002664; mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH), forward 5’-TGACCGGAAGCTCAGCTTG-3’, reverse 5’-TCCCACCCCTGTGCTGTA-3’, cDNA size 307 bp, and GenBank accession no. NM_001001303. After the RT step, PCRs were carried out in a real-time PCR cycler (Lightcycler; Roche Diagnostics) and analysed using Roche Molecular Biochemicals Lightcycler Software Version 3.5 (Roche Diagnostics). The optimized program involved denaturation at 95°C for 10 min, followed by 40 cycles of amplification (95°C for 10 s, 62°C for 15 s, 72°C for 15 s) in p47-phox and 95°C for 10 s, 60°C for 15 s, 72°C for 15 s in GAPDH). The temperature ramp rate was 20°C/s. At the end of each extension step, the fluorescence of each sample was measured to allow for the quantification of the PCR product. After completion of the PCR, the melting curve of each PCR product was measured to allow for the quantification of the PCR product. The enzyme reaction was started by adding tert-butyl hydroperoxide as a substrate and the 340 nm absorbance was recorded every 5 s for 1 min. The rate of decrease in the 340 nm absorbance was directly proportional to the GPX activity in the sample. The activity of SOD was measured using a SOD assay kit (Treivgen, Gaithersburg, MD). This method is based on the inhibition of the reduction of nitro blue tetrazolium by SOD. Superoxide ions convert nitro blue tetrazolium into blue formazan, which absorbs light at 550 nm. SOD reduces the superoxide ion concentration and thereby lowers blue formazan formation. The extent of reduction in the appearance of blue formazan reflects the amount of SOD activity in a sample.

**Light microscopic studies**

Obstructed or contralateral unobstructed kidneys were removed, fixed in 10% buffered formalin, and embedded in paraffin as described previously. Paraffin sections (3 μm thick) were stained using Masson trichrome and Azan-Mallory staining [8]. Two independent observers with no prior knowledge of the experimental design evaluated each tissue section using an Olympus light microscope (Olympus, Tokyo, Japan) with high-resolution digital camera systems (Penguin 600CL; Pixera Co., Los Gatos, CA, USA). Blue-stained interstitial fibrotic areas were observed, and images taken and analysed by means of an image analyser (Lumina Vision version 2.0; Mitani Corp., Tokyo, Japan). More than five fields were randomly selected in the tubulointerstitium and analysed in each section.

**Immunohistochemical studies**

Lipid peroxidation products and NADPH oxidase subunits were examined by immunoperoxidase staining as described previously [8]. Briefly, formalin-fixed, paraffin-embedded sections were deparaffinized, and endogenous peroxidase was inactivated with 0.3% H₂O₂. Sections were washed, preincubated in a blocking solution (10% goat serum in PBS) solution. The mixture was incubated at 25°C for 1 h, and the absorbance at 590 nm measured. GPX activity was determined by a method described previously with some modification [8]. The assay is an indirect measure of the activity of cellular GPX. Oxidized glutathione is recycled to its reduced form by glutathione reductase. The oxidation of NADPH resulting in NADP⁺ is accompanied by a decrease in absorbance at 340 nm so that GPX activity can be monitored. To assay GPX, tissue homogenate was added to a solution containing glutathione, glutathione reductase and NADPH. The enzyme reaction was started by adding tert-butyl hydroperoxide as a substrate and the 340 nm absorbance was recorded every 5 s for 1 min. The rate of decrease in the 340 nm absorbance was directly proportional to the GPX activity in the sample. The activity of SOD was measured using a SOD assay kit (Treivgen, Gaithersburg, MD). This method is based on the inhibition of the reduction of nitro blue tetrazolium by SOD. Superoxide ions convert nitro blue tetrazolium into blue formazan, which absorbs light at 550 nm. SOD reduces the superoxide ion concentration and thereby lowers blue formazan formation. The extent of reduction in the appearance of blue formazan reflects the amount of SOD activity in a sample.

**Renal catalase, glutathione peroxidase (GPX) and superoxide dismutase (SOD) activity**

When kidneys were harvested, each kidney was decapsulated, washed with saline, bisected coronally, blotted dry on gauze and weighed. After harvesting the obstructed or contralateral kidneys, samples were stored in a −80°C freezer until assay. Catalase activity was determined by measuring the removal rate of 70 μM H₂O₂ based on a method described previously [8]. Samples including the renal cortex were homogenized with a teflon homogenizer in homogenization buffer containing glutathione, glutathione reductase and NADPH. The oxidation of NADPH resulting in NADP⁺ is accompanied by a decrease in absorbance at 340 nm so that GPX activity can be monitored. The enzyme reaction was started by adding tert-butyl hydroperoxide as a substrate and the 340 nm absorbance was recorded every 5 s for 1 min. The rate of decrease in the 340 nm absorbance was directly proportional to the GPX activity in the sample. The activity of SOD was measured using a SOD assay kit (Treivgen, Gaithersburg, MD). This method is based on the inhibition of the reduction of nitro blue tetrazolium by SOD. Superoxide ions convert nitro blue tetrazolium into blue formazan, which absorbs light at 550 nm. SOD reduces the superoxide ion concentration and thereby lowers blue formazan formation. The extent of reduction in the appearance of blue formazan reflects the amount of SOD activity in a sample.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL)**

DNA fragmentation associated with apoptosis was detected in situ by the addition of nucleotides to free 3’ hydroxyl groups in DNA using MEBSTAIN Apoptosis Kit Direct (Medical and Biological Laboratories, Nagoya, Japan) as described previously [8]. More than 10 × 200 microscopic fields were examined in the animal at each time point, with the use of an Olympus immunofluorescence microscope. The number of fluorescent positive tubular epithelial cells was determined per field [8].

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Telmisartan inhibits renal fibrosis in acatalasemia

Telmisartan was found to inhibit renal fibrosis in acatalasemia. It was incubated with diaminobenzidine/H₂O₂ solution, counterstained with peroxidase (Nichirei Corp.) or cyanine 3 (Zymed Laboratories, Inc., South San Francisco, CA, USA), placed with streptavidin labelled type I or type IV collagen (Chemicon; dilution 1:50) for 1 h at room temperature. Each section was washed and incubated with rabbit polyclonal primary antibodies against type I or type IV collagen (Chemicon; dilution 1:50) for 1 h at room temperature. Each section was washed and incubated with the second antibody, FITC-conjugated goat anti-rabbit IgG (Zymed Laboratories, Inc.; dilution 1:80). After the section was washed, it was mounted with fluoromount-G.

Indirect immunofluorescence was performed as described previously [8]. Briefly, surgically removed kidney specimens were immediately snap-frozen and unfixed cryostat sections (4 μm thick) were prepared. The sections were washed and incubated with rabbit polyclonal primary antibodies against type I or type IV collagen (Chemicon; dilution 1:50) for 1 h at room temperature. Each section was washed and incubated with the second antibody, FITC-conjugated goat anti-rabbit IgG (Zymed Laboratories, Inc.; dilution 1:80). After the section was washed, it was mounted with fluoromount-G. FITC-positive tubulointerstitial areas were observed, and images taken and analysed using an image analyser (Lumina Vision version 2.0). More than 10 fields were randomly selected in the tubulointerstitium and analysed in each section.

Statistical analyses

Data, shown as mean±SEM, were analysed by one-way analysis of variance (ANOVA) with post-hoc multiple comparisons using the Scheffe method. Statistical analysis was performed using the StatView program (Hulinks, Tokyo, Japan). *P*<0.05 was taken to denote the presence of a statistically significant difference.

Results

Effect of telmisartan on arterial blood pressure in wild-type or acatalasemic UUO model

BW was similar between the wild-type and acatalasemic mice, and there were no significant fluctuations throughout the experiment in each group [8]. Mean blood pressure (MBP) was similar between the wild-type and acatalasemic mice at the start of the experiment (Table 1). The vehicle-treated controls showed that UUO does not affect MBP in wild-type or acatalasemic mice after 7 days. Upon intraperitoneal administration of telmisartan at 0.1 mg/kg BW per day, the level of MBP did not significantly decrease in either of the UUO mice after 7 days. However, a higher dose of telmisartan (0.3 mg/kg BW) significantly decreased MBP to below the normal blood pressure level after 7 days in both the wild-type and acatalasemic UUO mice compared with controls.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Group</th>
<th>Day 7</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>Control</td>
<td>108.8±3.8</td>
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<tr>
<td></td>
<td>UUO + vehicle buffer</td>
<td>109.4±3.4</td>
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<td></td>
<td>UUO + telmisartan 0.1 mg/kg</td>
<td>108.4±2.9</td>
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<td></td>
<td>UUO + telmisartan 0.3 mg/kg</td>
<td>98.5±3.8*</td>
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<tr>
<td>Acatalasemic</td>
<td>Control</td>
<td>109.8±4.9</td>
</tr>
<tr>
<td></td>
<td>UUO + vehicle buffer</td>
<td>109.3±2.4</td>
</tr>
<tr>
<td></td>
<td>UUO + telmisartan 0.1 mg/kg</td>
<td>108.8±4.1</td>
</tr>
<tr>
<td></td>
<td>UUO + telmisartan 0.3 mg/kg</td>
<td>98.8±2.8*</td>
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Data are means±SEM expressed in mmHg; *n* = 8 animals in each group.

*P*<0.05 vs wild-type control or UUO/vehicle buffer.

Telmisartan significantly attenuates renal interstitial fibrosis and deposition of collagens type I and type IV in acatalasemic obstructed kidneys

In the acatalasemic mice, the morphology of the major organs including the heart, lung, liver and pancreas appeared normal upon routine histological examination. The obstructed kidney (OBK) weight of wild-type mice showed no significant changes through day 7. However, the OBK weight of the acatalasemic mice significantly decreased on day 7 compared with control and was less than that of wild-type mice on day 7 (kidney/body weight ratio; wild-type, 0.77±0.08%; acatalasemic 0.62±0.05%; *P*<0.05). This reduction of renal mass in the acatalasemic mice was significantly suppressed by the treatment with 0.1 or 0.3 mg/kg BW of telmisartan (0.71±0.04% or 0.74±0.03%, respectively). The kidney of the acatalasemic mice exhibited normal morphology compared with that of wild-type mice by light microscopy (Figure 1A and D). The OBK of wild-type mice moderately developed obstructive nephropathy characterized by progressive tubulointerstitial changes (Figure 1B), while the acatalasemic OBK demonstrated significant tubulointerstitial injury including tubular dilation, atrophy, simplification of the tubular epithelium, increased number of fibroblasts and interstitial fibrosis as compared with the wild-type OBK (Figure 1E). Acatalasemic OBK displayed an enhanced deposition of collagens type I and IV in the tubulointerstitium compared with control kidneys or wild-type OBK (Figure 1G and H, J and K, M and N, P and Q). Treatment with telmisartan (0.1 mg/kg BW) not only significantly reversed the tubular atrophy and interstitial fibrosis (Figure 1C, F and S) but also dramatically reduced the deposition of tubulointerstitial collagens, even in the more fibrous acatalasemic OBK (Figure 1I, L, O, R, T and U).

Activation of NADPH oxidase subunits in obstructed kidneys of acatalasemic mice is significantly suppressed by telmisartan

Ang II-mediated ROS activation involves NADPH oxidases, which include a membrane-associated...
Fig. 1. Renal histology and deposition of tubulointerstitial collagens in wild-type or acatalasemic mice. Light micrographs of wild-type (A) or acatalasemic (D) control kidneys, wild-type (B) or acatalasemic (E) obstructed kidneys, and wild-type (C) or acatalasemic (F) obstructed kidneys treated by telmisartan (0.1 mg/kg BW) at day 7 are shown. Note marked tubular dilation, atrophy and interstitial expansion in acatalasemic obstructed kidneys significantly reversed by the treatment with telmisartan (0.1 mg/kg BW). Immunofluorescent micrographs of wild-type (G and M) or acatalasemic (J and P) control kidneys, wild-type (H and N) or acatalasemic (K and Q) obstructed kidneys, and wild-type (I and O) or acatalasemic (L and R) obstructed kidneys treated by telmisartan (0.1 mg/kg BW) at day 7 stained with type I (G–L) or type IV (M–R) collagens are shown. Note treatment with telmisartan (0.1 mg/kg BW) significantly suppressed deposition of type I and type IV collagens in tubulointerstitium of acatalasemic obstructed kidneys. Some perivascular staining of type I collagen in control kidneys is indicated by arrows (G and J). The morphometric analysis of fibrosis area in Masson-Trichrome stain (S) and positive area of collagens type I (T) or type IV (U) is shown. Each column consists of mean±SE, n=8 animals/group. #P<0.01 vs wild-type UUO at day 7. *P<0.05, **P<0.01 vs UUO in the same mouse group. †P<0.05 vs control in the same mouse group. W, wild-type mice; A, acatalasemic mice; UUO, unilateral ureteral obstruction; TS 0.1, telmisartan 0.1 mg/kg BW; 0.3, telmisartan 0.3 mg/kg BW. A–F: Masson-Trichrome stain. Scale bars=120μm.
subunit, p22-phox, and cytosolic subunits composed of p47phox and p67phox [5,6]. Immunohistochemical staining of these subunits demonstrated that normal wild-type and acatalasemic mouse kidneys expressed only trace amounts of the three subunits in tubular epithelial cells (Figure 2A, D, G, J, M and P). UUO significantly induced the expression of these subunits in the renal tubules of wild-type OBK on day 7 (Figure 2B, H and N). Greater atrophy of the tubules of the acatalasemic OBK yielded a more significant increase in expression of the subunits on day 7 (Figure 2E, K and Q). Morphometric analysis demonstrated that expression of p22-, p47- and p67-phox is activated in obstructed kidneys after UUO in both the wild-type and acatalasemic mice on day 7; however, the degree of activation is more severe in the acatalasemic OBK than wild-type OBK (Figure 2S–U). Treatment with telmisartan (0.1 mg/kg BW) dramatically suppressed the tubular expression of the NADPH oxidase subunits in both the wild-type (Figure 2C, I and O) and acatalasemic mice (Figure 2F, L and R) in a dose-dependent manner (Figure 2S–U). Using quantitative real-time PCR, we found that the acatalasemic OBK on day 7 after UUO had a significantly more abundant mRNA level for p22-, p47- and p67-phox compared with the wild-type OBK (Figure 3). This abundance was diminished by treatment with telmisartan.

Excessive lipid peroxidation in acatalasemic obstructed kidney is reduced by telmisartan

The generation of ROS modifies proteins and lipids in the cell membrane in tissue and may play a crucial role in a variety of biological activities such as cell apoptosis and extracellular matrix accumulation. We next examined whether acatalasemia-induced lipid peroxidation products could be reduced by telmisartan. There was an increase in the expression of HNE (Figure 4) and HHE in dilated tubules of the wild-type and acatalasemic OBK [8]. More intense expression was observed in the cytoplasm of the dilated tubules in acatalasemic OBK as reported previously [8]. Morphometric analysis by digital densitometry revealed that the percentage of positive areas for both peroxidation products was significantly reduced by the treatment with telmisartan in a dose-dependent manner (Figure 4).

Telmisartan inhibits acatalasemia-induced sensitization of tubular epithelial cells to apoptosis in obstructed kidney

Cell loss by apoptosis plays an important role in the pathogenesis of the renal tubular atrophy associated with renal fibrosis in UUO [8]. The frequency of tubular epithelial cell apoptosis was >90% of the total positive cells in acatalasemia and peaked on day 4 after UUO in our setting [8]. Therefore we investigated whether telmisartan could inhibit tubular cell apoptosis at this time point (Figure 5). The TUNEL technique quantitatively demonstrated a significant decrease in the number of cells positive for apoptosis following the administration of telmisartan in both the wild-type and acatalasemic OBK (Figure 5C).

Treatment with telmisartan did not change the level of antioxidant enzymes in obstructed kidney

The catalase activities of the major organs are as follows: wild-type liver, 34.3 ± 9.1; acatalasemic liver 13.5 ± 2.1; wild-type kidney, 23.0 ± 0.8; acatalasemic kidney 3.9 ± 0.1; wild-type lung, 3.4 ± 0.3; acatalasemic lung 0.9 ± 0.2; wild-type heart, 1.4 ± 0.4; acatalasemic heart 0.2 ± 0.2 (nmol/s/mg protein). It was necessary to remove residual blood by perfusion of the kidneys with saline, since moderate amounts of catalase activity are found in erythrocytes (catalase activity of wild-type haemolysate, 5.57 ± 0.95; acatalasemic haemolysate, 0.99 ± 0.29 nmol/s/mg Hb). Renal catalase activity in the acatalasemic control mice exhibited a significant decrease compared with wild-type control mice, and it remained low throughout the experiment (Table 2). The renal catalase activity in wild-type mice significantly decreased on day 7 after UUO. To examine the effect of acatalasemia on other renal antioxidant enzymes in the fibrosing kidney, we investigated the activity of GPX and SOD in the OBK of wild-type or acatalasemic mice (Table 2). There was no compensatory upregulation of GPX or SOD in acatalasemic OBK. Treatment of telmisartan did not affect any of the renal antioxidant enzymes examined (Table 2).
Discussion

A number of randomized clinical trials have strongly suggested that the AT1 receptor antagonists and/or the angiotensin-converting enzyme inhibitors possess potential renoprotective effects [1]. However, the mechanism whereby the blockade of the renin-angiotensin system inhibits renal fibrosis has not been
fully elucidated thus far. Since ROS are associated with many of the Ang II signalling pathways, we investigated whether the AT1 receptor antagonist telmisartan could inhibit oxidant-mediated tissue injury and suppress renal fibrosis after UUO in acatalasemic mice. Telmisartan significantly inhibited the expression of collagen type I and type IV in tubulointerstitium, and of the NADPH oxidase subunits p22phox, p47phox and p67phox. In addition, lipid peroxidation products, apoptosis in the tubular epithelium, and subsequent renal interstitial fibrosis in OBK were all reduced. This was true under acatalasemic conditions as well as the normal catalase environment.

Oxidative stress results from an excessive production of ROS, an impaired antioxidant system or a combination of both. The acceptance of oxygen carrying one electron, as occurs during electron transport of mitochondria, is affected by enzymes including xanthine oxidase, NADPH oxidase and cycloxygenase, and yields the superoxide anion (O$_2^-$). This anion, in turn, is converted to hydrogen peroxide by the action of SOD. The source of ROS in tissue injury is largely dependent on the types of enzymes activated. Our recent study utilizing acatalasemic mice demonstrated that excess ROS induced by functional catalase deficiency sensitized tubular epithelial cells to apoptosis, oxidant tissue injury and renal fibrosis after UUO [8]. Xanthine oxidase has been implicated in tissue injury following ischaemia-reperfusion because of its ability to generate ROS under these conditions. We thus examined the activity of xanthine oxidase in the OBK of wild-type or acatalasemic mice as a source of ROS; however, there were no significant increases in xanthine oxidase activity in two mice groups [8]. In the current study, a strong induction of NADPH oxidase subunit expression in the dilated tubules of wild-type and acatalasemic OBK was observed (Figure 2). Therefore this enzyme, rather than xanthine oxidase, may be the major source of ROS in UUO-mediated renal fibrosis, which occurs in acatalasemia.

NADPH oxidase consists of a membrane-associated cytochrome b$_{558}$, composed of a p22phox subunit and a gp91phox subunit, as well as at least four cytosolic subunits that include p47phox, p67phox, p40phox and the small GTPases rac1 and rac2 [5,6]. Superoxide, hydrogen peroxide and hydroxyl radicals are generated after phosphorylation of the cytosolic p47phox subunit, which plays a crucial role in agonist (Ang II, PMA and TNFα)-induced NADPH oxidase activation [5,6]. Ang II has been reported to upregulate the synthesis of NADPH oxidase in various cell types, including...
glomerular mesangial cells in vitro. The expression of NADPH oxidase and its subunits within the kidney may give insight into Ang II-mediated ROS generation. Components of NADPH oxidase have also been identified in cultured mouse proximal tubular epithelial cells [10], glomerular cells in Dahl salt-sensitive rats [11], glomerular endothelial cells, podocytes, distal and collecting tubules, vessels, and interstitial fibroblasts in the kidney of 10-week-old spontaneously hypertensive rats [9].

The degree of oxidative stress and the severity of subsequent renal fibrosis may depend on an imbalance between excessive production of ROS and antioxidant defence within the kidney. Catalase is a major enzyme that catalyzes the decomposition of hydrogen peroxide (H₂O₂) and plays a role in cellular antioxidant defence mechanisms [12]. This enzyme is localized in the matrix of peroxisomes in mammalian cells and is involved in two different types of enzymatic reaction: the catalatic activity (2H₂O₂ → O₂ + 2H₂O) and the peroxidatic activity (H₂O₂ + AH₂ → A + 2H₂O). Catalase thus limits the accumulation of H₂O₂, which is generated by various oxidases in tissue and serves as a substrate for the Fenton reaction to generate the highly injurious hydroxyl radical. Takahara [13] first documented genetic defects of catalase in Japanese patients who exhibited a deficiency of blood catalase enzyme activity (acatalasemia). Short term clinical manifestations in acatalasemic patients after exposure to H₂O₂ or infection with peroxide-generating bacteria such as streptococci and pneumococci appear predominantly in the mouth with the result of an increase in activity of phagocytic cells at the inflamed sites. Subsequently, Feinstein et al. [14] established an acatalasemic mouse strain (Csb) from the progeny of X-ray-irradiated mice. They screened blood catalase levels and developed congenic control (C3H/AnL/Csa) and acatalasemic (C3H/AnL/Csb) mouse strains. The tissues of acatalasemic mice express normal catalase mRNA levels compared with those of wild-type mice, suggesting the mutation does not act at the level of gene transcription or mRNA stability, but rather at the level of mRNA translation and/or protein turnover. The mutation has been mapped to the mouse catalase structural gene on chromosome 2 (Cat or Cas1) and is expressed by modification of the enzyme active site but not of the antigenic site [14]. Shaffer and Preston [15] identified a Gln to His substitution at amino acid position 11 (G to T transversion) in acatalasemic catalase cDNA. Since this amino acid is located within a region that

**Fig. 4.** Quantification of lipid peroxidation products in renal tubulointerstitium in obstructed kidneys of wild-type or acatalasemic mice. The level of lipid peroxidation products including HNE- (A–G) or HHE-modified proteins (H) in tubulointerstitium of obstructed kidneys were detected by immunohistochemistry and quantified by morphometric analysis. Positive areas were quantified by digital morphometry. Each column consists of mean±SE, n=8 animals/group. #P<0.05 vs wild-type UUO at day 7. *P<0.05. **P<0.01 vs UUO in the same mice group. †P<0.05 vs control in the same mice group. W, wild-type mice; A, acatalasemic mice; TS 0.1, telmisartan 0.1 mg/kg BW; 0.3, telmisartan 0.3 mg/kg BW. Scale bars=120 μm.
forms the first major α-helix in the N-terminal arm of the catalase subunit, this may render the catalase molecule unstable in acatalasemic mice [15]. Wang et al. [16] cloned the cDNA of catalase from a hypocatalasemic mouse strain (Csc) and reported that it contains a missense point mutation at amino acid position 439, which leads to an Asp to Ser substitution (A to G transversion). No obvious abnormalities in the histology or function of the kidneys are observed in acatalasemic mice under normal conditions. Acatalasemic mice are fertile and show no apparent developmental defects. However, enhanced incidence of liver tumours is reported in these mice treated with diethylnitrosamine, suggesting that the increased oxidative stress burden brought about by acatalasemia may be involved in hepatocarcinogenesis [17]. A new line of catalase-null mice has been generated recently by a gene targeting technique [18]. However, the mouse phenotype of a complete Cat gene knockout may not accurately reflect the human acatalasemic conditions, since patients with acatalasemia retain residual catalase activity in tissues.

We found that treatment with a low dose (0.1 mg/kg BW) of telmisartan significantly inhibited expression of NADPH oxidase subunits, peroxidation products, and renal fibrosis without lowering the systemic blood pressure, although we did not utilize intra-arterial measurements or radiotelemetry recordings of the blood pressure. However, telmisartan did not change the level of renal antioxidant enzymes. This finding forms the first major α-helix in the N-terminal arm of the catalase subunit, this may render the catalase molecule unstable in acatalasemic mice [15]. Wang et al. [16] cloned the cDNA of catalase from a hypocatalasemic mouse strain (Csc) and reported that it contains a missense point mutation at amino acid position 439, which leads to an Asp to Ser substitution (A to G transversion). No obvious abnormalities in the histology or function of the kidneys are observed in acatalasemic mice under normal conditions. Acatalasemic mice are fertile and show no apparent developmental defects. However, enhanced incidence of liver tumours is reported in these mice treated with diethylnitrosamine, suggesting that the increased oxidative stress burden brought about by acatalasemia may be involved in hepatocarcinogenesis [17]. A new line of catalase-null mice has been generated recently by a gene targeting technique [18]. However, the mouse phenotype of a complete Cat gene knockout may not accurately reflect the human acatalasemic conditions, since patients with acatalasemia retain residual catalase activity in tissues. 

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Table 2. Activities of renal anti-oxidant enzymes in UUO model

<table>
<thead>
<tr>
<th>Mice</th>
<th>Group</th>
<th>Catalase</th>
<th>Glutathione peroxidase</th>
<th>Superoxide dismutase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Control</td>
<td>23.0±0.8</td>
<td>2.6±0.3</td>
<td>700±45</td>
</tr>
<tr>
<td></td>
<td>UUO + vehicle buffer</td>
<td>6.1±0.2</td>
<td>2.1±0.2*</td>
<td>612±23^</td>
</tr>
<tr>
<td></td>
<td>UUO + telmisartan 0.1 mg/kg</td>
<td>6.5±0.5^</td>
<td>2.0±0.1*</td>
<td>607±45^</td>
</tr>
<tr>
<td></td>
<td>UUO + telmisartan 0.3 mg/kg</td>
<td>6.3±0.4^</td>
<td>2.1±0.2*</td>
<td>616±32^</td>
</tr>
<tr>
<td>Acatalasemic</td>
<td>Control</td>
<td>3.9±0.1</td>
<td>2.6±0.4</td>
<td>676±36</td>
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<td>UUO + vehicle buffer</td>
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<td>2.0±0.1b</td>
<td>603±21b</td>
</tr>
<tr>
<td></td>
<td>UUO + telmisartan 0.1 mg/kg</td>
<td>3.8±0.1</td>
<td>1.9±0.2b</td>
<td>612±19b</td>
</tr>
<tr>
<td></td>
<td>UUO + telmisartan 0.3 mg/kg</td>
<td>3.9±0.1</td>
<td>2.1±0.1b</td>
<td>608±18b</td>
</tr>
</tbody>
</table>

Data are means±SEM expressed in nmol/s/mg at day 7; n=8 animals in each group.

*P<0.05 vs wild-type control.

bP<0.05 vs acatalasemic control.

Fig. 5. Tubular epithelial cell apoptosis in obstructed kidneys of wild-type or acatalasemic mice. Fluorescent micrographs of TUNEL-positive tubular epithelial cells in acatalasemic obstructed kidneys (A and B) or acatalasemic obstructed kidneys treated by telmisartan (0.1 mg/kg BW) at day 4 (C) are shown. The positive cells are shown in dilated tubules (arrows) as well as in tubular lumen (arrowheads). The effects of telmisartan on the level of apoptosis at day 4 after UUO is shown in (D). #P<0.01 vs wild-type UUO at day 7. *P<0.05, **P<0.01 vs UUO in the same mouse group. †P<0.05 vs control in the same mice group. W, wild-type mice; A, acatalasemic mice; TS 0.1, telmisartan 0.1 mg/kg BW; 0.3, telmisartan 0.3 mg/kg BW. Scale bars = 50 μm.
suggests that telmisartan inhibits the generation of ROS rather than effecting ROS detoxification, possibly via attenuation of the local renin–angiotensin system. Yang et al. [19] demonstrated that Ang II blockade by losartan alone partially, but combination with hepatocyte growth factor gene therapy significantly suppressed renal interstitial fibrosis after UUO in mice. Since several AT1 receptor antagonists differ in their pharmacological profile and chemical structure, the differences may particularly affect their efficacy as antioxidants rather than as antihypertensive agents. Recent reports further suggest that telmisartan has structural similarities to the PPARγ ligand pioglitazone, and thus could act as a partial agonist of PPARγ to decrease oxidative stress [20].

In conclusion, the AT1 receptor antagonist telmisartan significantly inhibited the expression of the NADPH oxidase p22/p47phox subunits, a source of ROS, and lipid peroxidation products, including HNE and HHE, in the tubulointerstitium of acatalasemic mice after UUO. This was achieved without any effect on the activity of other antioxidant enzymes. Telmisartan also decreases the frequency of apoptosis in tubular epithelial cells, the expression of collagen type I and type IV in tubulointerstitium and, finally, in the level of renal interstitial fibrosis, even under acatalasemic conditions. These inhibitory effects, though dose dependent, were independent of a decrease in systemic blood pressure. Further elucidation of the antioxidant and antifibrotic mechanisms of AT1 receptor antagonists such as telmisartan will yield important new insights into the treatment of renal disease.

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Conflict of interest statement. None declared.

References

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