Renal Liver-Type Fatty Acid Binding Protein Attenuates Angiotensin II–Induced Renal Injury

Daisuke Ichikawa, Atsuko Kamijo-Ikemori, Takeshi Sugaya, Takashi Yasuda, Seiko Hoshino, Jyunko Igarashi-Migitaka, Kazuaki Hirata, Kenjiro Kimura

Abstract—To investigate the role of human liver-type fatty acid binding protein (hL-FABP) in angiotensin (Ang) II–induced renal injury, Ang II was infused systemically into hL-FABP chromosomal transgenic (Tg) and wild-type (WT) mice (Tg-Ang II and WT-Ang II) for 28 days. Control mice were injected with saline only (Tg-control and WT-control). hL-FABP was expressed in proximal tubules of Tg mice. After a high-dose injection of Ang II, renal gene and protein expressions of hL-FABP in Tg-Ang II mice increased significantly compared with Tg-control mice. Urinary excretion of L-FABP was significantly greater in Tg-Ang II than in Tg-control mice. Blood pressure levels in both groups increased to a similar extent. Upregulation of monocyte chemoattractant protein 1 expression, macrophage infiltration in the interstitium, tubulointerstitial damage, and depositions of type I and III collagens were observed in both Tg-Ang II and WT-Ang II mice. However, these effects were less pronounced in Tg-Ang II compared with WT-Ang II mice. The level of renal N-(hexanoyl)lysine, an oxidative stress marker, was significantly higher in WT-Ang II than in Tg-Ang II mice. In conclusion, renal hL-FABP reduced oxidative stress in Ang II–induced renal injury and attenuated tubulointerstitial damage.

Key Words: angiotensin II ■ L-type fatty acid binding protein ■ oxidative stress ■ hypertension ■ tubulointerstitial damage

The number of patients with hypertensive nephrosclerosis has been increasing as a result of an aging society and an increasing prevalence of metabolic syndrome. Tubulointerstitial damage is strongly associated with progression of renal dysfunction, and relief of tubulointerstitial damage leads to its inhibition. Angiotensin (Ang) II, which is generated by activation of the renin-Ang system, causes tubulointerstitial damage via generation of oxidative stress, and it aggravates the progression of renal dysfunction. Therefore, a protein or agent with an antioxidative function may inhibit the progression of renal injury.

LIVER-TYPE FATTY ACID BINDING PROTEIN (L-FABP) IS A 14-KDA PROTEIN FOUND IN THE CYTOSPLASM OF HUMAN PROXIMAL TUBULES. L-FABP CAN BIND WITH HIGH AFFINITY TO LONG-CHAIN FATTY ACID OXIDATION PRODUCTS AND MAY BE AN EFFECTIVE ENDOGENOUS ANTIOXIDANT. RENAL L-FABP IS RARELY EXPRESSED IN RODENT KIDNEYS. To elucidate its pathophysiological role in kidney disease, we generated human L-FABP (hL-FABP) chromosomal transgenic (Tg) mice. However, the pathophysiological significance of hL-FABP remained to be determined in renal injury induced by Ang II.

Systemic Ang II infusion into mice is well known to induce glomerular sclerosis and tubulointerstitial damage in the medulla of the kidney via a hypertension effect of Ang II causing vasoconstriction. However, renal injury in the cortex is rarely observed. Ang II exerts numerous physiological responses through specific receptors located on plasma membranes. There are 2 different types of mammalian Ang II receptors, Ang II type 1 (AT1) and Ang II type 2, and these responses are mediated primarily through AT1 receptors. A massive dose into mice of Ang II, which is an agonist of the AT1 receptor, causes moderate tubulointerstitial damage and slight glomerular sclerosis in the cortex. The purpose of this study was to assess the renoprotective effect of renal hL-FABP in systemic Ang II infusion mice.

Materials and Methods

Animals

Studies were conducted in accordance with the St Marianna University School of Medicine Institutional Guide for Animal Experiments. Because renal L-FABP is not expressed in the mouse kidney, hL-FABP Tg mice were generated as described previously (World Intellectual Property Organization patent WO0073791). Eight to 10-week-old male hL-FABP Tg mice (n=35; body weight, 23.7±0.2 g) on a C57/BL6 background and wild-type (WT) mice purchased from Japan SLC Inc (Shizuoka, Japan; n=36; body weight, 23.4±0.3 g) were used.
Model of Ang II–Induced Renal Damage
As an Ang II model, we used an agonist of the AT1 receptor, Val5-Ang II acetate salt hydrate (Val5-Ang II; A2900, Sigma-Aldrich Co), in which the fifth amino acid of an Ang II octapeptide was changed from isoleucine to valine. Both the Tg and WT mice were divided into 2 groups; the Ang II group (Tg-Ang II, n=23; WT-Ang II, n=23) received systemic Val5-Ang II infusion (5 µg/kg per minute; Sigma-Aldrich Co) using an osmotic minipump (Alzet model 1004, Durect Corp). Val5-Ang II was dissolved in sterile saline and implanted via the osmotic minipump into the SC space of mice anesthetized with isoflurane. The control group (Tg-control, n=12; WT-control, n=12) was given only saline.

Blood Pressure
Blood pressure was measured through a tail-cuff apparatus (Softeron BP-98A, Softeron Co, Ltd, Tokyo, Japan) every week after implantation of the osmotic minipump. Systolic blood pressure values were derived from an average of 3 measurements per animal at each time point.

Serum and Urinary Biochemistry
Serum creatinine was measured by the Jaffé method (The Creatinine Companion, Exocell). For urine collection on days 0, 14, and 28, all of the mice were housed overnight individually in metabolic cages with free access to tap water. Urine parameters are reported as ratios to urinary creatinine levels. Urinary creatinine was measured by the Jaffé method (The Creatinine Companion,). Urinary N-acetyl-β-D-glucosaminidase was quantified using a commercially available chemical reagent (NAGtest, Shionogi, Osaka, Japan). Albuminuria was determined using the Albwueil assay (Exocell). Urinary hL-FABP was measured by a 2-step sandwich ELISA procedure (human L-FABP ELISA kit, CMIC Co, Ltd, Tokyo, Japan).

Renal Histological and Morphometric Analysis
For light microscopic analysis, the kidneys were dehydrated and embedded in paraffin. Serial sections (2-µm thick) were obtained for conventional histological assessment, such as periodic acid-Schiff staining and Azan-Mallory staining, and for immunohistochemistry. Tubulointerstitial injury was categorized as tubular dilation with epithelial atrophy and extracellular matrix accumulation in periodic acid-Schiff–stained tissue sections. Azan-Mallory–stained tissue sections were used to evaluate tubulointerstitial fibrosis, which was defined as accumulation of extracellular matrix (staining blue) and tubular atrophy. Under magnification (>200), 10 nonoverlapping fields from both the cortical and the medullary areas were selected, and the area of tubulointerstitial injury or fibrosis, as well as the entire cortical and medullary area, were measured with image analyzer version 6.1 (WinRoof, Mitani Co, Tokyo, Japan). Each degree of tubulointerstitial injury and of fibrosis were evaluated as ratios to the entire cortical or medullary area.11,12 For glomerulosclerosis quantification, the grade of sclerosis in each glomerulus stained with periodic acid-Schiff was defined as described previously.13

Immunohistological Analysis
Tissues fixed in methyl Carnoy solution were embedded in paraffin. An indirect immunoperoxidase method was used to identify the antigens, as described previously.11,12 Macrophages were identified using the rat monoclonal antibody F4/80 (BMA Biomedicals, Augst, Switzerland), and type I or type III collagens were identified using rabbit polyclonal antibodies (Cedarlane Laboratories). Ten nonoverlapping fields from both the cortical and medullary areas were selected. The degree of macrophage infiltration in the cortical or medullary interstitium was expressed as the ratio of the positive area of F4/80 to the entire cortical or medullary area under ×200 magnification, measured with an image analyzer (WinRoof). Similarly, the positive areas for type I and type III collagen were expressed as ratios of the positive areas for type I and type III collagens to the entire cortical or medullary area.

Measurement of Renal Ne-(Hexanoyl)Lysine by ELISA
To determine the quantity of Ne-(hexanoyl)Lysine (HEL) proteins in the kidney, the protein extracted by the method described above was measured with an ELISA kit for HEL (Japan International Cooperation Agency, Shizuoka, Japan).

Statistical Analysis
All of the values were expressed as mean±SE. Statistical significance was set at P<0.05. To compare parameters of the 2 groups, the Mann-Whitney U test using unpaired data were used.

Results
Blood Pressure in Response to Ang II
Systolic blood pressure of the Ang II group in Tg-Ang II was similar to that of WT-Ang II from days 7 to 28 (Figure 1).

Serum and Urinary Biochemistry
The serum creatinine level did not differ significantly between Ang II and control mice in the Tg and WT mice or between Tg-Ang II and WT-Ang II mice (Table). Urinary N-acetyl-β-D-glucosaminidase levels in Tg-Ang II mice were significantly lower than in WT-Ang II mice on
pathophysiological role of renal L-FABP in Ang II

975

Urinary albumin levels did not differ significantly between Tg-Ang II and WT-Ang II mice (Table).

Dynamics of hL-FABP Expression in the Kidney

The gene expression levels of renal hL-FABP (Figure 2A) in Tg-Ang II mice on days 14 (P<0.01) and 28 (P<0.01) were significantly higher than in Tg-control mice on the same day. The protein expression levels of renal hL-FABP (Figure 2B) in Tg-Ang II mice on days 14 (P<0.01) and 28 (P<0.01) were significantly higher than in Tg-control mice on the same day. Urinary hL-FABP levels were significantly higher in Tg-Ang II mice than in Tg-control mice on days 14 (P<0.05) and 28 (P<0.01; Figure 2C).

In the double immunohistochemical staining of hL-FABP and aquaporin 1 of the Tg-control mice kidney, many double-positive tubules (hL-FABP+/aquaporin-1+) were observed in the cortical area. In Tg-Ang II mice on days 14 and

Table. Serum and Urinary Biochemistry Findings in hL-FABP transgenic and WT Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control WT Mice</th>
<th>Control Tg Mice</th>
<th>Ang II WT Mice</th>
<th>Ang II Tg Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine, mg/dL</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 14</td>
<td>0.91±0.06</td>
<td>1.01±0.05</td>
<td>1.08±0.05</td>
<td>1.14±0.06</td>
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<tr>
<td>Day 28</td>
<td>1.04±0.03</td>
<td>1.12±0.05</td>
<td>1.03±0.03</td>
<td>1.06±0.03</td>
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<tr>
<td>Urinary albumin, µg/mg of creatinine</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>5.0±2.0</td>
<td>2.8±1.1</td>
<td>4.1±0.9</td>
<td>3.1±0.5</td>
</tr>
<tr>
<td>Day 14</td>
<td>4.7±2.0</td>
<td>4.2±2.1</td>
<td>10.8±3.9*‡</td>
<td>7.3±1.9*‡</td>
</tr>
<tr>
<td>Day 28</td>
<td>1.1±0.1</td>
<td>0.9±0.1</td>
<td>13.4±1.5*‡</td>
<td>11.3±2.9*‡</td>
</tr>
<tr>
<td>Urinary NAG, U/mg of creatinine</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>0.072±0.009</td>
<td>0.074±0.013</td>
<td>0.084±0.011</td>
<td>0.072±0.008</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.077±0.013</td>
<td>0.070±0.009</td>
<td>0.123±0.005‡</td>
<td>0.074±0.007†</td>
</tr>
<tr>
<td>Day 28</td>
<td>0.068±0.003</td>
<td>0.085±0.017</td>
<td>0.108±0.013*‡</td>
<td>0.118±0.014‡</td>
</tr>
</tbody>
</table>

NAG indicates N-acetyl-β-D-glucosaminidase; hL-FABP, human liver-type fatty acid binding protein; WT, wild-type; Ang, angiotensin.

*P<0.05 vs the control group on the same day.
†P<0.05 vs the WT group on the same day.
‡P<0.05 vs the same group on day 0.

Figure 2. Dynamics of human liver-type fatty acid binding protein (hL-FABP) in the kidney. A, Expression of hL-FABP mRNA transcripts. *P<0.01. B, Expression of hL-FABP protein. *P<0.01 vs the control group on the same day. C, Time course of urinary hL-FABP levels. †P<0.01 vs the same group on day 0. Double immunohistochemical staining of hL-FABP (purple) and aquaporin-1 (brown) in the kidney cortex and medulla (D), cortex (E), and juxtamedulla (F) of transgenic (Tg)-control mice on day 14 and of Tg-angiotensin (Ang) II mice on day 14. Original magnification, ×40 (D); ×200 (E and F).
Figure 3A. Expression of monocyte chemoattractant protein 1 (MCP-1) in the kidney. A, Expression of MCP-1 mRNA transcripts. B, Expression of MCP-1 protein. Data are reported as mean±SE. "P<0.05 vs the control group on the same day; †P<0.05 vs the wild-type (WT) group on the same day; §P<0.05 vs the same group on day 14; ¶P<0.05 vs the medulla group on the same day.

Expression of MCP-1 in the Kidney
The gene expression levels of MCP-1 in Tg-Ang II mice on days 14 (P<0.05) and 28 (P<0.05) were significantly lower than in WT-Ang II mice measured on the same day (Figure 3A). The protein expression levels of MCP-1 in Tg-Ang II mice on days 14 (P<0.01) and 28 (P<0.01) were significantly lower than those measured in WT-Ang II mice on the same day (Figure 3B).

Evaluation of Macrophage Infiltration
The macrophage infiltration on the cortex of the kidneys in the Tg-Ang II mice on days 14 (P<0.01) and 28 (P<0.01) was significantly inhibited than in the WT-Ang II mice on the same days (Figure 4).

Renal Histological and Morphometric Analysis
In both the Tg-Ang II and WT-Ang II mice, periodic acid–Schiff–stained sections revealed tubulointerstitial damage, including dilatation of tubules and degeneration of proximal tubular epithelial cells on days 14 and 28 (Figure 5), and Azan–Mallory–stained sections revealed tubulointerstitial fibrosis (blue staining; Figure 6). The tubulointerstitial damage areas of the cortex in Tg-Ang II mice were significantly lower than in WT-Ang II mice on days 14 (P<0.01) and 28 (P<0.01; Figure 5C). The glomerular sclerosis scores were not significantly different among all of the groups (Figure 5D). The levels of tubulointerstitial fibrosis in Azan–Mallory–stained sections of the kidney in the cortex of Tg-Ang II mice were significantly lower than in WT-Ang II mice on days 14 (P<0.01) and 28 (P<0.01; Figure 6B).

Immunohistological Analysis of Type I and Type III Collagen
Deposition levels of type I and type III collagens on the cortex of the kidneys in Tg-Ang II mice on day 14 (P<0.01) and 28 (P<0.01) were significantly lower than in WT-Ang II mice measured on the same day (Figure 7A and 7C). The protein expression levels of α-1 type I collagen in Tg-Ang II mice on days 14 (P<0.01) and 28 (P<0.01) were significantly lower than those measured in WT-Ang II mice on the same day (Figure 7B).

Gene Expression of α-1 Type I Collagen and α-1 Type III Collagen in Kidney
The gene expression levels of α-1 type I collagen (Figure 8A) and α-1 type III collagen (Figure 8B) in the kidneys of Tg-Ang II mice on day 14 were significantly lower than in the kidneys of WT-Ang II mice on the same day (P<0.05).

Evaluation of Oxidative Stress
In the experimental model of Ang II–induced nephropathy used in this study, we have demonstrated that the expression of renal hL-FABP was upregulated in Tg-Ang II mice, and urinary excretion of N-acetyl-β-d-glucosaminidase from the proximal tubules and the production of MCP-1, α-1 type I collagen, and α-1 type III collagen were suppressed. An early oxidative stress marker, renal HEL accumulation, was significantly higher in theWT-Ang II mice than in the Tg mice on day 14 (Figure 9). The number of infiltrating macrophages and the deposition of type I...
type III collagens were significantly lower in the cortex of the Tg-Ang II kidneys than in the WT-Ang II kidneys (Figures 4 and 7). Tubulointerstitial damage of the cortex was significantly attenuated in the Tg-Ang II kidneys versus the WT-Ang II kidneys (Figure 5). These results suggested the possibility that renal hL-FABP inhibited fibrosis and production of inflammatory cytokines and attenuated tubulointerstitial damage of the Ang II infusion model via reduction of oxidative stress.

In our systemic Ang II infusion model, tubulointerstitial damage and glomerular sclerosis were observed in both the cortex and the medulla. In the medullary tubulointerstitial damage and the juxtamedullary glomeruli injury, hypertension-induced vascular damage by vasoconstriction of Ang II plays an important role. Hypertensive vascular damage occurs first in the juxtamedullary glomeruli, leading to tubular hypoxia provoked by impairment of renal medullary circulation. This occurs downstream from the juxtamedullary glomeruli and consequently leads to medullary tubulointerstitial damage. The cortex is reported to be well protected from pressure-induced injury because renal cortical blood flow exhibits a high degree of autoregulation, and cortical renal damage is rarely found. Therefore, the tubulointerstitial damage and glomerular sclerosis in the cortex were considered to be induced as a direct effect of Ang II rather than by elevation of the blood pressure. Ang II activates the AT1 receptor, which is expressed not only in the glomeruli but also in the tubules.

Figure 5. Histological findings of periodic acid-Schiff (PAS). Magnification, ×200 in A. Magnification, ×400 in B through D. The figure in A illustrates the tubulointerstitial damage. Normal glomerulus and glomerular sclerosis are shown in B. Each area was also assessed quantitatively (C and D), as described in the Materials and Methods section. "c" shows the cortex, "jm" shows the juxtamedulla, and "m" shows the medulla. Values are expressed as mean±SE. *P<0.01 vs the control group on the same day; †P<0.05 vs the wild-type (WT) group on the same day; ‡P<0.05 vs the same group on day 14; §P<0.05 vs the medulla group.

Figure 6. Histological findings of Azan-Mallory staining (A). Magnification, ×200. Each area was also assessed quantitatively (B) as described in the Materials and Methods section; “c” indicates the cross-axle expression in the cortex, and “m” the cross-axle expression in the medulla in B; values are mean±SE. *P<0.05 vs the control group on the same day; †P<0.05 vs the wild-type (WT) group on the same day; ‡P<0.05 vs the same group on day 14; §P<0.05 vs the medulla group.
where it elevates the level of intracellular reactive oxygen species, increases oxidative stress to the glomeruli and the tubules, and causes renal cortical damage. Moreover, cortical glomerular damage leads to tubular hypoxia via reduction of postglomerular blood flow, and this can progress until tubulointerstitial damage occurs.

HEL is formed by oxidative stress modification including oxidation of omega 6 fatty acids, such as linoleic acid or arachidonic acid. In the WT-Ang II mice, renal HEL levels, an early oxidative stress marker, sharply increased on day 14. Because renal hL-FABP has an antioxidative function in the proximal tubules of Tg-Ang II mice, renal HEL levels and cortical tubulointerstitial damage (Figure 9) were significantly suppressed compared with those measured in WT-Ang II mice. These results suggested that progression of cortical tubulointerstitial damage was strongly associated with the degree of oxidative stress.

Renal hL-FABP was upregulated in the proximal tubules of Tg-Ang II mice (Figure 2). We reported previously that renal hL-FABP expression was upregulated in various experimental models in which oxidative stress contributes to severe tubulointerstitial damage. Therefore, the onset of oxidative stress generated in Tg-Ang II mice might also promote expression of renal hL-FABP in Ang II–induced nephropathy. Moreover, the results of double immunohistochemistry revealed that hL-FABP expression was observed in the straight portion of the proximal tubules (S3 segment) in addition to the convoluted proximal tubules (Figure 2). Because damaged tubules in the Ang II–induced nephropathy model are involved in the S3 segment tubules, de novo expression of hL-FABP in the S3 segment of the proximal tubules might be upregulated to prevent tubulointerstitial damage.

The degree of glomerulosclerosis and the medullar tubulointerstitial damage, which is composed of not only proximal tubules but also thin segments of the loop of Henle, distal tubules, and collecting tubules, were not improved in the

Figure 7. Immunoisotological staining using an antibody against type I collagen (A) and an antibody against type III collagen (B). Original magnification, ×200. These areas were also assessed quantitatively (C and D), as described in the Materials and Methods section. Data are reported as mean±SE. *P<0.05 vs the control group on the same day; †P<0.05 vs the wild-type (WT) group on the same day; ‡P<0.05 vs the same group on day 14; §P<0.05 vs the medulla group on the same day.

Figure 8. Gene expression of (A) α-1 type I collagen (COLIA1) mRNA transcripts and (B) α-1 type III collagen (COLIIIA1) mRNA transcripts in the kidney. Data are reported as mean±SE. *P<0.05 vs the control group on the same day; †P<0.05 vs the wild-type (WT) group on the same day.
Tg-Ang II mice (Figures 4–7). Because hL-FABP is expressed in the proximal tubules that occupy most of the cortex, the cortex tubulointerstitial damage was ameliorated in the Tg-Ang II mice compared with the WT-Ang II mice but not glomerular damage and medullary tubulointerstitial damage. To our knowledge, this is the first observation that hL-FABP attenuated cortical tubulointerstitial damage in an experimental model in which cortical and medullary tubulointerstitial damage were induced by different mechanisms in the same kidney.

Subcutaneously injected Ang II was absorbed into the systemic circulation where it is loaded into the proximal tubules via AT1 receptors. The blood pressure (Figure 1) and gene expression levels of the AT1a receptor (data not shown) in the kidneys of Tg-Ang II and WT-Ang II mice over the experimental periods showed no significant differences. Ureapulation of Hypoxia-inducible factor 1α gene expression indicates the hypoxic stress loaded on the proximal tubular cell. Because hypoxia-inducible factor 1α gene expression in the Tg-Ang II mice was similar to that in the WT-Ang II mice (Figure S1, available in the online-only Data Supplement), the degree of tubular hypoxia induced by Ang II–induced vascular damage was not different between these 2 groups. Therefore, it was speculated that the amount of Ang II loaded on the kidneys was equivalent in the 2 groups.

In the Ang II–induced nephropathy model, hypertension is forcibly induced via vasoconstriction of Ang II. Therefore, the results that attenuation of renal injury led to blood pressure lowering or progression of renal injury increased systemic blood pressure in this model were not found.

**Perspectives**

In clinical practice with patients having nephrosclerosis, inhibition of the renin-Ang system is often recommended to prevent progression of nephrosclerosis. However, because this medication alone cannot completely suppress the progression of nephrosclerosis, more effective protective treatments are needed. In our Ang II infusion model, cortical tubulointerstitial damage was provoked, and renal hL-FABP suppressed the progression of cortical tubulointerstitial damage. Although the degree of glomerular sclerosis was not improved by attenuation of cortical tubulointerstitial damage in this study, agents that upregulated the expression of renal L-FABP in the proximal tubules might serve as important therapeutic targets to prevent cortical tubulointerstitial damage in nephrosclerosis.

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**Disclosures**

None.

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### Novelty and Significance

**What Is New?**
- hL-FABP expressed in proximal tubules was upregulated in Ang II–induced renal damage.
- hL-FABP suppressed tubulointerstitial damage in the cortex.

**What Is Relevant?**
- An important complication of hypertension, nephrosclerosis, is not prevented by inhibiting the renin-Ang system.

**Summary**
Renal hL-FABP reduced oxidative stress in Ang II–induced renal injury and attenuated tubulointerstitial damage.
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ONLINE SUPPLEMENT:
RENAL L-TYPE FATTY ACID BINDING PROTEIN ATTENUATES ANGIOTENSIN II-INDUCED RENAL INJURY

Daisuke Ichikawa, Atsuko Kamijo-Ikemori, Takeshi Sugaya, Takashi Yasuda, Seiko Hoshino, Jyunko Igarashi-Migitaka, Kazuaki Hirata and Kenjiro Kimura
Mice were housed in the animal facilities of St. Marianna University School of Medicine with free access to food and water before this experiment. The dose of [Val$^5$]-Ang II had been determined in a preliminary study. We evaluated the ability of different doses of [Val$^5$]-Ang II (2 or 5ug/kg/min) to induce renal injury in male C57/BL6 wild-type mice purchased from Japan SLC Inc (Shizuoka, Japan). Only a slight degree of cortical tubulointerstitial damage in the mice injected with 2ug/kg/min [Val$^5$]-Ang II for 28 days was observed. The mice injected with 5ug/kg/min showed moderate tubulointerstitial injury including infiltration of macrophages, tubular dilatation and interstitial fibrosis. Therefore, injection of 5ug/kg/min [Val$^5$]-Ang II was performed in this study. After removing the kidneys of these groups under intraperitoneal anesthesia on days 14 and 28, blood was drawn from the inferior vena cava. The left kidney was then removed, fixed in 10% buffered formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and stained with methyl Carnoy’s solution. The right kidney was removed, and snap-frozen in liquid nitrogen for analysis of protein and for gene expression assays.

The control group (Tg- control, $n = 12$; WT- control, $n = 12$) was given only saline. The kidneys of these groups were removed on day 14 (Tg- AngII, $n = 13$; WT-AngII, $n = 13$; Tg- control, $n = 6$; WT- control, $n = 6$) and on day 28 (Tg- AngII, $n = 10$; WT- Ang II, $n = 10$; Tg- control, $n = 6$; WT- control, $n = 6$).

Additional Figures and Supporting Information

1) Gene Expression of Hypoxia Inducible Factor-1$\alpha$ (HIF1$\alpha$) in the Kidney

Method

Total RNA was extracted and was reverse-transcribed as described previously. The TaqMan real-time PCR reaction was performed. The mRNAs of hypoxia inducible factor-1$\alpha$ (HIF1$\alpha$) were detected. The expression levels of those mRNAs in each sample were normalized using GAPDH expression levels.

Results

The HIF1$\alpha$ gene expression levels did not differ between Tg-Ang II and WT-Ang II (Figure S1).

2) Correlation between urinary hL-FABP and Histological changes

Method

Statistical Analysis; The correlation between urinary hL-FABP and histological
change was analyzed by Spearman’s rank coefficient of correlation.

Results

In the combined Tg-Ang II and Tg-control groups, urinary hLFABP was significantly correlated with tubulointerstitial damage of both cortical and medulla (Cortex; Figure S2A, r = 0.41, P < 0.05, Medulla; r = 0.40, P < 0.05), the degree of tubulointerstitial fibrosis of both cortical and medulla (Cortex; Figure S2B, r = 0.65, P < 0.01, Medulla; r = 0.45, P < 0.05), F4/80 positive area per field in the cortical interstitium (Cortex; Figure S2C, r = 0.51, P < 0.01, Medulla; r = 0.58, P = 0.07), the level of deposition of type I collagen in the cortical interstitium (Cortex; Figure S2D, r = 0.44, P < 0.05, Medulla; r = 0.37, P = 0.40) and the level of deposition of type III collagen in both cortical and medullar regions (Cortex; Figure S2E, r = 0.41, P < 0.05, Medulla; r = 0.67, P < 0.05). These results indicated that urinary hL-FABP was a useful biomarker for tubulointerstitial injury.

3) Localization of AT1a receptor and renal hL-FABP in the kidney

Method

In rodents, AT1 receptors exist as two isoforms, designated AT1a and AT1b, at two different loci that are 94% identical at the amino acid level and are pharmacologically indistinguishable from each other. The AT1a receptor is expressed in most tissues including the kidney, plays a major role in the renal actions of Ang II and is the murine homologue to the single human AT1 receptor. Therefore, in order to evaluate the localization of both the AT1a receptor and renal hL-FABP in the kidney, we generated mutant mice in which the AT1a receptor gene was disrupted by replacing it with the β-galactosidase (LacZ) gene such that LacZ activity was under transcriptional control of the endogenous AT1a locus, and renal hL-FABP was expressed in the proximal tubules. The homozygous mutant mice with an AT1a gene disruption described previously and the hL-FABP Tg mice described above were interbred to obtain heterozygous mutant mice that were AT1a gene deficient and expressed renal hL-FABP (Ko-Tg mice). Only male mice at the age of eight weeks were used. The mice were divided into two groups: the Ko-Tg Ang II group (n=8) was infused with [Val5]-Ang II as described above and the Ko-Tg control group (n=5) was infused with saline for 28 days. On day 28 after the start of infusion, the kidneys were removed and fixed in a solution containing 2% paraformaldehyde, 0.2% glutaraldehyde, and 0.02% Nonidet P-40 in phosphate-buffered saline for 60 min at room temperature, stained at 4°C overnight in a solution containing 0.4mg/ml Bluo-Gal (Life Technologies, Inc. Carlsbad, CA) for LacZ staining, and re-fixed in a solution of 4%
paraformaldehyde at 4°C overnight. The tissues were then dehydrated and embedded in paraffin. Serial sections (2 μm thick) were obtained for immunohistochemistry with monoclonal antibody hL-FABP, against which the peroxidase was developed using 3,3-Diaminobenzidine to evaluate its expression as described previously. Nuclear staining was performed with nuclear fast red staining. In the previous study, LacZ staining was reported to be identified not only in the glomerulus, juxtaglomerular apparatus of the renal cortex, but also in the proximal tubules of the renal cortex.

Results

The Ko-Tg Ang II group had significantly higher SBP than the control group from days 7 to 28 (P < 0.01). SBP remained unchanged in the Ko-Tg control mice (Figure S3A). LacZ staining was identified not only in the glomerulus, juxtaglomerular apparatus of the renal cortex, but also in the proximal tubules of the renal cortex, with hL-FABP staining in both the Ko-Tg control and Ko-Tg Ang II mice kidney (Figure S3B). In Ko-Tg control mice, double-positive tubules (hL-FABP+/LacZ+) were observed in the cortical area; in Ko-Tg Ang II mice on day 28, double-positive tubules were found not only in the cortex but also in the medulla. These results indicated that hL-FABP expression was observed in the straight portion of the proximal tubules (S3 segment) in addition to the convoluted proximal tubules, which was consistent with the area where expression of the AT1a receptor was observed. Because damaged tubules in the Ang II-induced nephropathy model are involved in the S3 segment tubules, de novo expression of hL-FABP in the S3 segment of the proximal tubules might be up-regulated to prevent tubulointerstitial damage.

Reference of supplement


**Figure S1**

Gene expression of HIF1α in the kidney. Data are reported as means ± SE. *P < 0.05* versus the control group on the same day.

**Figure S2**

Significant correlation between urinary hL-FABP and tubulointerstitial damage by PAS staining (A), the degree of tubulointerstitial fibrosis by Azan-Mallory staining (B), F4/80 positive area per field in the interstitium by immunohistological staining using an antibody against F4/80 (C) and the deposition of type I and III collagen by immunohistological staining using an antibody against type I collagen and an antibody against type III collagen (D, and E) in the combined Tg-Ang II and Tg-control group.
Figure S3

A

![Graph showing systolic blood pressure over time](image)

B

![Images of cortex and medulla](image)

Time-related changes in systolic blood pressure (A) and localization of AT1a receptor and renal hL-FABP (B) in the Ko-Tg Control and Ko-Tg Ang II groups. A: Systolic blood pressure rose significantly in response to Ang II infusion in the Ko-Tg mice. Values are mean ± SE. *P < 0.01, compared with the control group on the same day; †P < 0.01, compared with the same group on day 0. B: Double immunohistochemical staining of hL-FABP (brown) and LacZ (blue) in the kidney cortex and medulla of the Ko-Tg control mice and of Ko-Tg Ang II mice on day 28. Original magnification: x200.