Renoprotective effect of renal liver-type fatty acid binding protein and angiotensin II type 1a receptor loss in renal injury caused by RAS activation

Daisuke Ichikawa,1 Atsuko Kamijo-Ikemori,1,2 Takeshi Sugaya,1 Yugo Shibagaki,1 Takashi Yasuda,1 Kimie Katayama,1 Seiko Hoshino,2 Junko Igarashi-Migitaka,2 Kazuaki Hirata,1,2 and Kenjiro Kimura1

1Division of Nephrology and Hypertension, Department of Internal Medicine, St. Marianna University School of Medicine, Kanagawa, Japan; and 2Department of Anatomy, St. Marianna University School of Medicine, Kanagawa, Japan

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The renin-angiotensin system (RAS) plays a central role in the progression of chronic kidney disease (CKD) (1, 6, 23), inhibitors of RAS activation, such as with angiotensin-converting enzyme inhibitor or angiotensin II (ANG II) receptor blocker, are considered to be the first line of treatment for patients with CKD to suppress the progression of the disease (12, 26). However, these medications do not completely inhibit the progression of CKD (2) and new strategies using other mechanisms apart from inhibition of RAS activation are needed.

Liver-type fatty acid binding protein (L-FABP) is expressed in human renal proximal tubules (16). L-FABP is known to work as an effective endogenous antioxidant during oxidative stress generated in pathophysiological conditions (25, 29, 30). Because renal L-FABP is not endogenously expressed in the kidneys of mice, we generated human L-FABP (hL-FABP) chromosomal transgenic mice and evaluated the pathophysiological role of renal hL-FABP (8) in the transgenic mice. Results of animal model studies of kidney disease showed that hL-FABP mRNA expression in the kidney was upregulated by stress, such as urinary protein overload (8), tubular ischemia (30), tubular stretch (9), hyperglycemia (10), and toxins (17, 32), which in turn causes tubulointerstitial damage, and that this tubulointerstitial damage could be attenuated via reducing oxidative stress.

The RAS is strongly activated by high-salt intake in addition to increases in ANG II levels (11, 14), and this activation is involved in severe tubulointerstitial damage (11). In a model of RAS activation, the expression of renal hL-FABP was reported to increase in the proximal tubules with ANG II type 1a receptor (AT1a) expression and attenuated the tubulointerstitial damage via reduction of oxidative stress (7, 21). The aim of this study was to clarify the renoprotective function of renal hL-FABP in renal injury due to RAS activation and its role in reduction of AT1a, which is expressed in most tissues including the kidney (27) and plays a major role in the renal actions of ANG II (4, 5).

MATERIALS AND METHODS

Animals

Studies were conducted in accordance with the St. Marianna University School of Medicine Institutional Guide for Animal Experiments. Male C57/BL6 wild-type mice (L-FABP+/+AT1a+/+) were purchased from Japan SLC (Shizuoka, Japan). hL-FABP chromosomal transgenic mice of a C57/BL6 background (L-FABP+/+AT1a+/+) were generated previously (27). To obtain L-FABP+/+AT1a−/− mice, the L-FABP+/+AT1a+/+ mice were crossed with AT1a−/− mice without the hL-FABP transgene (L-FABP+/+AT1a−/−). After the next back-cross of these mice, L-FABP+/+AT1a−/− mice were obtained.

L-FABP+/−AT1a+/+ mice and L-FABP+/−AT1a−/− mice were littermates of the L-FABP+/+AT1a+/+ and L-FABP+/−AT1a−/− mice, respectively. Eight- to ten-week-old male mice were used for...
experiments, L-FABP+/+AT1a+/+ mice (n = 16; mean body weight, 25.5 ± 0.5 g), L-FABP+/+AT1a+/− mice (n = 15; mean body weight, 26.1 ± 0.8 g), L-FABP+/+AT1a−/− mice (n = 18; mean body weight, 27.2 ± 0.5 g), L-FABP−/−AT1a+/+ mice (n = 18; mean body weight, 24.0 ± 0.2 g), L-FABP−/−AT1a+/− mice (n = 12; mean body weight, 25.2 ± 0.7 g), and L-FABP−/−AT1a−/− mice (n = 15; mean body weight, 27.6 ± 0.5 g) were used.

**Mouse Model of Renal Damage Induced by RAS Activation**

We used an agonist of the AT1 receptor, [Val5]-ANG II (no. A2900; Sigma-Aldrich, St. Louis, MO), in which the fifth amino acid of an ANG II octapeptide was changed from isoleucine to valine (Val) (7). L-FABP+/+AT1a+/+ mice (n = 10), L-FABP+/+AT1a+/− mice (n = 10), L-FABP+/+AT1a−/− mice (n = 10), L-FABP−/−AT1a+/+ mice (n = 7), and L-FABP−/−AT1a+/− mice (n = 10) were administered systemic [Val5]-ANG II by infusion (5 μg·kg−1·min−1) using an osmotic minipump (Alzet model 1004; Durect) and were given 5% high-salt diet for 28 days (7). The selection of [Val5]-ANG II and the dose of [Val5]-ANG II were previously determined in a preliminary study. We evaluated the difference between normal ANG II and [Val5]-ANG II and the ability of different doses of [Val5]-ANG II (2 or 5 μg·kg−1·min−1) to induce renal injury in male C57BL/6 wild-type mice. Mice injected with the normal ANG II or 2 μg·kg−1·min−1 [Val5]-ANG II for 28 days showed only a slight degree of cortical tubulointerstitial damage. The mice injected with 5 μg·kg−1·min−1 showed moderate tubulointerstitial injury including infiltration of macrophages, tubular dilatation, and interstitial fibrosis. Therefore, a dose of 5 μg·kg−1·min−1 [Val5]-ANG II was chosen for this study. These mice are referred to as L-FABP+/+AT1a+/+RAS, L-FABP+/+AT1a+/−RAS, L-FABP+/+AT1a−/−RAS, L-FABP−/−AT1a+/+RAS, L-FABP−/−AT1a+/−RAS, and L-FABP−/−AT1a−/−RAS. [Val5]-ANG II was dissolved in sterile saline and infused via an osmotic minipump that was implanted into the subcutaneous tissue of mice while anesthetized with isoflurane. Control mice were given only saline (L-FABP+/+AT1a+/+control, L-FABP+/+AT1a+/−control, L-FABP−/−AT1a+/+control, L-FABP−/−AT1a+/−control, L-FABP−/−AT1a−/−control).

After the kidneys of these groups were removed while the animals were under intraperitoneal anesthesia on day 28, blood was drawn from the inferior vena cava. The left kidney was then removed and fixed in 10% buffered formalin (Wako Pure Chemical Industries, Augst, Switzerland), and type I and type III collagens were identified using a rabbit polyclonal antibodies (Cedarlane Laboratories). Ten nonoverlapping fields from the cortical areas were selected. The degree of macrophage infiltration in the cortical interstitium was assessed as a ratio relative to the entire cortical area (7). The degree of macrophage infiltration in the cortical interstitium was expressed as the ratio of the positively stained area of F4/80 relative to the entire cortical 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 positively stained areas for type I and type III collagens relative to the entire cortical area.

**Blood Pressure**

Blood pressure was measured in conscious, restrained mice through a tail-cuff apparatus (Softron BP-98A; Softron, Tokyo, Japan) every week after implantation of the osmotic minipump for 28 days (7). Systolic blood pressure (SBP) values were derived from an average of three measurements per animal at each time point of days 7, 14, 21, and 28. Blood pressure of the mice was measured twice a week before this experimental study to obtain an accurate value of blood pressure.

**Serum and Urinary Biochemistry**

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. The sediment was removed from the urine samples by centrifugation (15,000 rpm for 5 min). The mice were put overnight in the metabolic cages 1 wk before the study to acclimatize the mice to the cage environment.

Serum and urinary creatinine were measured by an enzymatic method (Nescoat VL II CRE; Alfresa Pharma, Osaka, Japan) (7). Urinary parameters are reported as ratios relative to urinary creatinine levels. Albuminuria was determined using the Albuwell assay (Exocell, PA). Urinary L-FABP was measured by a two-step sandwich ELISA (hL-FABP ELISA kit; CMIC, Tokyo, Japan) (8).

**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 assessments, such as periodic acid-Schiff staining, and for immunohistochemistry. Tubulointerstitial injury was categorized as tubular dilatation with epithelial atrophy and extracellular matrix accumulation in periodic acid-Schiff-stained tissue sections. Under magnification (×200), 10 nonoverlapping fields from the cortical areas were selected, and the area of tubulointerstitial injury or fibrosis, as well as the entire cortical area, was measured with image analyzer version 6.1 (Winroof; Mitani, Tokyo, Japan). The degree of tubulointerstitial injury and of fibrosis in each case was expressed as a ratio relative to the entire cortical area (7).

For quantitation of glomerulosclerosis, the severity of sclerosis in each glomerulus stained with periodic acid-Schiff was graded as described previously (7) and expressed as a ratio relative to its grade in the corresponding control group.

**Immunohistochemical Analysis**

Tissues fixed in methyl Carnoy solution were embedded in paraffin. An indirect immunoperoxidase method was used to identify the antigens, as described previously (17, 32). Macrophages were identified using the rat monoclonal antibody F4/80 (BMA Biomicals, Augst, Switzerland), and type I and type III collagens were identified using a rabbit polyclonal antibodies (Cedarlane Laboratories). Ten nonoverlapping fields from the cortical areas were selected. The degree of macrophage infiltration in the cortical interstitium was expressed as the ratio of the positively stained area of F4/80 relative to the entire cortical 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 positively stained areas for type I and type III collagens relative to the entire cortical area.

**In Situ Detection of Reactive Oxygen Species Production**

To evaluate reactive oxygen species (ROS) production of the kidneys in situ, unfixed frozen cross-sections from the kidney were stained with 10 μM dihydroethidium (DHE) (Invitrogen, Carlsbad, CA) for 30 min in a dark humidified chamber at 37°C. ROS generation was detected as a red fluorescence and visualized by fluorescence microscopy (13, 15). The DHE fluorescence intensities of kidney cross sections were examined in five nonoverlapping fields from the cortical areas and were expressed as the ratio of the positively stained area of DHE relative to the entire cortical area under ×200 magnification, measured with an image analyzer (Winroof).

**Measurement of Monocyte Chemoattractant Protein 1 and hL-FABP by ELISA**

Proteins were extracted from frozen kidney, and protein concentration was measured as described previously (7). Monocyte chemotactrant protein-1 (MCP-1) and hL-FABP were measured by ELISA (MCP-1 from R&D Systems and h-L-FABP from CMIC) (7). The concentrations of MCP-1 and h-L-FABP were corrected for total protein concentrations.

**Real-Time Quantitative Polymerase Chain Reaction Analysis**

Total RNA was extracted and was reverse transcribed as described previously (17, 32). The TaqMan real-time polymerase chain reaction (PCR) reaction was performed using a TaqMan ABI PRISM 7000 sequence detection system (Applied Biosystems). Real-time PCR was used to measure the mRNAs of h-L-FABP, MCP-1, α1-type I collagen,
α1-type III collagen, and GAPDH. The expression levels of these mRNAs in each sample were normalized to GAPDH expression levels.

**Statistical Analysis**

All of the values were expressed as means ± SE. Statistical significance was set at P < 0.05. Mann-Whitney U-test for unpaired data was used to compare the measurements between two groups.

**RESULTS**

**Study 1: Renoprotective Function of Renal hL-FABP and Decrease in AT1a Expression**

mRNA expression of AT1a in kidneys. The mRNA levels of AT1a in the kidney of knockout mice (AT1a+/− and AT1a−/− mice) were significantly lower than those of wild-type mice (L-FABP−/−AT1a+/+) and L-FABP+/−AT1a+/+ mice (L-FABP−/−AT1a+/+ control, 2.5 ± 1.0 vs. L-FABP−/−AT1a+/+ control, 0.7 ± 0.1, P < 0.05; L-FABP−/−AT1a+/+RAS, 1.9 ± 0.6 vs. L-FABP−/−AT1a+/+RAS, 0.5 ± 0.1, P < 0.05; L-FABP−/−AT1a+/+RAS, 3.2 ± 0.7 vs. L-FABP+/−AT1a+/+RAS, 1.0 ± 0.3, P < 0.05; and L-FABP+/−AT1a+/+RAS, 3.0 ± 0.6 vs. L-FABP+/−AT1a+/+RAS, 1.3 ± 0.4, P < 0.05). mRNA expression values are in arbitrary units. The mRNA expression levels of AT1a in the kidneys of L-FABP+/− mice were similar to those of L-FABP−/− mice (Fig. 1). In both L-FABP+/− and L-FABP−/− mice, there were no significant differences in the mRNA levels of AT1a between RAS and control groups.

**Blood pressure in response to RAS activation.** From days 7 to 28, there were similar significant SBP increases in both L-FABP−/−AT1a+/+RAS and L-FABP+/−AT1a+/+RAS mice, compared with that seen in both L-FABP−/−AT1a+/+ and L-FABP+/−AT1a+/+ controls (P < 0.05). In the same period (7–28 days), SBP in the AT1a+/+RAS and AT1a−/−RAS was significantly less elevated than in the AT1a+/+RAS. On day 28, the values (mmHg) were as follows: L-FABP−/−AT1a+/+RAS, 110.5 ± 2.1 vs. L-FABP+/−AT1a+/+RAS, 130.4 ± 2.8 vs. L-FABP−/−AT1a+/+RAS, 158.2 ± 7.6, P < 0.05; and L-FABP−/−AT1a−/−RAS, 112.3 ± 1.9 vs. L-FABP−/−AT1a−/−RAS, 133.9 ± 3.9 vs. L-FABP+/−AT1a−/−RAS, 152.3 ± 4.6, P < 0.05 (Fig. 2). SBP levels of the L-FABP+/− mice were similar to those of L-FABP−/− mice.

**Serum and urinary biochemistry.** Serum creatinine levels were not significantly different between RAS and control mice in L-FABP−/− and L-FABP+/− mice or between L-FABP−/−RAS and L-FABP+/−RAS mice regardless of AT1a expression (Table 1).

On day 28, the urinary albumin levels of the AT1a+/+RAS mice (with L-FABP−/− and L-FABP+/− genotypes) and AT1a−/−RAS mice (with L-FABP−/− and L-FABP+/− genotypes) were significantly lower than those of the AT1a+/+RAS mice (with L-FABP−/− and L-FABP+/− genotypes; P < 0.05). Urinary albumin levels of the L-FABP−/−RAS mice (with AT1a+/+, AT1a−/+), and AT1a−/−) were similar to those of L-FABP−/−RAS mice (with AT1a+/+, AT1a−/+, and AT1a−/−) genotype on days 14 and 28, the values were as follows (expressed as μg/mg creatinine): L-FABP−/−AT1a+/+RAS, 50.6 ± 17.4 vs. L-FABP−/−AT1a+/+RAS, 53.0 ± 7.3, P = 0.28; L-FABP+/−AT1a+/+RAS, 22.7 ± 7.6 vs. L-FABP−/−AT1a+/+RAS, 20.0 ± 2.6, P = 0.22; and L-FABP+/−AT1a−/−RAS, 0.8 ± 0.2 vs. L-FABP+/−AT1a+/−RAS, 0.6 ± 0.1, P = 0.27 (Fig. 3). In both L-FABP−/− controls and L-FABP+/− controls, urinary albumin levels on days 14 and 28 were similar to those on day 0 (data not shown).

**Expression of MCP-1 in the kidney.** The mRNA levels of MCP-1 (Fig. 4A) in L-FABP−/−AT1a+/+RAS, L-FABP−/−AT1a+/+RAS, and L-FABP+/−AT1a+/+RAS mice were significantly higher than those in the control mice (P < 0.05). L-FABP+/− (AT1a+/+RAS and AT1a−/−RAS) and L-FABP−/− mice (AT1a+/+RAS and AT1a−/−RAS) had significantly lower MCP-1 mRNA expression levels than the L-FABP−/−AT1a+/+RAS and L-FABP+/−AT1a+/+RAS mice (P < 0.05). mRNA levels in the L-FABP−/−AT1a+/+RAS and L-FABP+/−AT1a+/+RAS mice were significantly lower than those in the L-FABP−/−AT1a+/+RAS mice.

**Table 1. Serum biochemistry findings in L-FABP−/− and L-FABP+/− Mice**

<table>
<thead>
<tr>
<th>Parameters/Genotype of AT1a</th>
<th>L-FABP−/− Control Mice</th>
<th>L-FABP−/− RAS Mice</th>
<th>L-FABP−/− Control Mice</th>
<th>L-FABP−/− RAS Mice</th>
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<tbody>
<tr>
<td>Serum creatinine, mg/dl</td>
<td></td>
<td></td>
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<tr>
<td>AT1a+/+</td>
<td>0.17 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.20 ± 0.07</td>
</tr>
<tr>
<td>AT1a−/−</td>
<td>0.13 ± 0.02</td>
<td>0.17 ± 0.04</td>
<td>0.15 ± 0.01</td>
<td>0.20 ± 0.01</td>
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Values are means ± SE. L-FABP, liver-type fatty acid binding protein; AT1a, angiotensin II type 1A receptor; RAS, renin-angiotensin system.
AT1a+/−RAS and L-FABP−/−AT1a+/−RAS mice (L-FABP+/−AT1a+/−RAS, 0.3 ± 0.1 vs. L-FABP+/−AT1a+/−RAS, 1.1 ± 0.4, P < 0.05, and L-FABP−/−AT1a+/−RAS, 2.8 ± 1.1 vs. L-FABP−/−AT1a+/−RAS, 4.5 ± 0.6, P < 0.05; values in arbitrary units).

The protein expression levels of MCP-1 (Fig. 4B) in L-FABP−/−AT1a+/−RAS, L-FABP−/−AT1a+/−RAS, and L-FABP−/−AT1a+/−RAS mice were significantly higher than those in the control mice (P < 0.05). L-FABP+/− and L-FABP+/− mice (AT1a+/−RAS and AT1a−/−RAS) had significantly lower MCP-1 protein expression levels than that of the L-FABP−/−AT1a+/−RAS and L-FABP−/−AT1a+/−RAS mice (P < 0.05). Protein levels in the L-FABP+/−AT1a+/−RAS and L-FABP+/−AT1a+/−RAS mice were significantly lower than those of L-FABP+/−AT1a+/−RAS and L-FABP+/−AT1a+/−RAS mice (L-FABP+/−AT1a+/−RAS, 4.8 ± 0.8 vs. L-FABP+/−AT1a+/−RAS, 10.0 ± 2.5 pg/mg protein, P < 0.05; and L-FABP+/−AT1a+/−RAS, 11.3 ± 2.4 vs. L-FABP+/−AT1a+/−RAS, 20.0 ± 4.4 pg/mg protein, P < 0.05).

Evaluation of macrophage infiltration. The macrophage infiltration in the cortex of the kidneys in L-FABP+/−AT1a+/−RAS, L-FABP−/−AT1a+/−RAS, and L-FABP−/−AT1a+/−RAS mice was significantly higher than that in the control mice (P < 0.05; Fig. 5). In L-FABP+/− and L-FABP+/− (AT1a−/−RAS and L-FABP+/− AT1a+/−RAS) mice, the degree of macrophage infiltration was significantly lower than that of the L-FABP−/− AT1a+/−RAS and L-FABP−/− AT1a+/−RAS mice (P < 0.05). The degree of macrophage infiltration in the L-FABP+/− AT1a+/−RAS and L-FABP+/− AT1a+/−RAS mice was significantly lower than that in the L-FABP−/− AT1a+/−RAS and L-FABP−/− AT1a+/−RAS mice (L-FABP+/− AT1a+/−RAS, 0.07 ± 0.01 vs. L-FABP+/− AT1a+/−RAS, 0.12 ± 0.05%, P < 0.05; and L-FABP+/− AT1a+/−RAS, 0.16 ± 0.03 vs. L-FABP−/− AT1a+/−RAS, 0.27 ± 0.04%, P < 0.05).

Renal histological and morphometric analysis. The kidney cortical areas showing tubulointerstitial damage in L-FABP−/− AT1a+/−RAS, L-FABP−/− AT1a+/−RAS, L-FABP+/− AT1a+/−RAS, and L-FABP−/− AT1a+/−RAS mice were significantly more severe than those in the control mice (P < 0.05; Figs. 6B and 7B). In FABP+/− and FABP+/− (AT1a+/−RAS and AT1a−/−RAS) mice, the areas of tissue damage were significantly smaller than those of the FABP−/− AT1a+/−RAS and FABP+/− AT1a+/−RAS mice (P < 0.05). The areas of damage in the L-FABP+/− AT1a+/−RAS and L-FABP+/− AT1a+/−RAS mice were significantly smaller than those in the L-FABP+/− AT1a+/−RAS and L-FABP+/− AT1a+/−RAS mice (L-FABP+/− AT1a+/−RAS, 1.7 ± 0.3% vs. L-FABP+/− AT1a+/−RAS, 3.9 ± 0.9%, P < 0.05; and L-FABP+/− AT1a+/−RAS, 4.2 ± 0.8% vs. L-FABP+/− AT1a+/−RAS, 10.5 ± 0.6%, P < 0.05).

Although the glomerular sclerosis scores were not significantly different among the various groups, the scores in L-FABP+/− AT1a+/−RAS, L-FABP+/− AT1a+/−RAS, L-FABP+/− AT1a−/−RAS, and L-FABP+/− AT1a+/−RAS mice tended to be lower than those of the FABP−/− AT1a+/−RAS and FABP+/− AT1a+/−RAS mice. The scores of the L-FABP+/− AT1a+/−RAS mice were similar to those of L-FABP−/− AT1a+/−RAS mice (Fig. 7).

Immunohistological analysis of type I and type III collagen. The extent of deposition of type I collagen (Figs. 5C and 6C) and type III collagen (Figs. 5D and 6D) in the cortex in L-FABP−/− AT1a+/−RAS, L-FABP−/− AT1a+/−RAS, and L-FABP+/− AT1a+/−RAS mice was significantly higher than those in the control mice (P < 0.05). In L-FABP+/− and L-FABP+/− (AT1a+/−RAS and AT1a−/−RAS) mice, the extent of deposition of type I and Type III collagens was significantly lower than in L-FABP+/− AT1a+/−RAS and L-FABP+/− AT1a+/−RAS mice (P < 0.05). Deposition levels in the L-FABP+/− AT1a+/−RAS mice were significantly lower than in the L-FABP−/− AT1a+/−RAS mice (type I collagen,
L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, 1.2 ± 0.2% vs. L-FABP<sup>−/+</sup> AT1a<sup>+/+</sup> RAS, 2.2 ± 0.2%, P < 0.05; and type III collagen, L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, 0.9 ± 0.2% vs. L-FABP<sup>−/+</sup> AT1a<sup>+/+</sup> RAS, 1.4 ± 0.2%, P < 0.05).

mRNA expression levels of α<sub>1</sub>-type I collagen and α<sub>1</sub>-type III collagen in the kidney. The mRNA levels of α<sub>1</sub>-type I collagen (Fig. 8A) in the L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, L-FABP<sup>−/+</sup> AT1a<sup>+/+</sup> RAS, and L-FABP<sup>+/+</sup> AT1a<sup>+/+</sup> RAS mice were significantly higher than those in the control mice (P < 0.05). In L-FABP<sup>−/−</sup> and L-FABP<sup>−/+</sup> (AT1a<sup>−/−</sup> RAS and AT1a<sup>+/+</sup> RAS) mice, mRNA expression levels were significantly lower than in L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS mice (P < 0.05). mRNA expression levels in the L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS and L-FABP<sup>−/+</sup> AT1a<sup>+/+</sup> RAS mice were significantly lower than those in the control mice (0.5 ± 0.1 vs. L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, 1.3 ± 0.2, P < 0.05; and L-FABP<sup>−/+</sup> AT1a<sup>+/+</sup> RAS, 1.1 ± 0.2 vs. L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, 1.9 ± 0.3, P < 0.05; values in arbitrary units).

The mRNA levels of α<sub>1</sub>-type III collagen (Fig. 8B) in L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, L-FABP<sup>−/+</sup> AT1a<sup>+/+</sup> RAS, L-FABP<sup>+/+</sup> AT1a<sup>+/+</sup> RAS, and L-FABP<sup>+/−</sup> AT1a<sup>+/+</sup> RAS mice were significantly higher than those in the control mice (P < 0.05). In the L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS mice, mRNA levels were significantly lower than L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS mice (P < 0.05). In the L-FABP<sup>+/−</sup> AT1a<sup>+/+</sup> RAS and AT1a<sup>−/−</sup> RAS mice, mRNA levels were significantly lower than those of the L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS and L-FABP<sup>+/−</sup> AT1a<sup>+/+</sup> RAS mice (P < 0.05). mRNA levels in the L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, L-FABP<sup>−/+</sup> AT1a<sup>+/+</sup> RAS, and L-FABP<sup>+/+</sup> AT1a<sup>+/+</sup> RAS mice were significantly lower than in the L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, L-FABP<sup>−/+</sup> AT1a<sup>+/+</sup> RAS, and L-FABP<sup>+/−</sup> AT1a<sup>+/+</sup> RAS mice (L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, 0.8 ± 0.2 vs. L-FABP<sup>−/+</sup> AT1a<sup>+/+</sup> RAS, 3.6 ± 0.9, P < 0.05; and L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, 1.4 ± 0.3 vs. L-FABP<sup>−/+</sup> AT1a<sup>+/+</sup> RAS, 4.0 ± 0.5, P < 0.05; values in arbitrary units).

**Evaluation of oxidative stress.** The production of ROS was examined by DHE staining in kidney sections. DHE fluorescence was significantly greater in the kidneys of L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS and L-FABP<sup>+/−</sup> AT1a<sup>+/+</sup> RAS mice than in those of the corresponding control mice (P < 0.05). In L-FABP<sup>−/−</sup> and L-FABP<sup>+/−</sup> (AT1a<sup>−/−</sup> RAS and AT1a<sup>−/−</sup> RAS) mice, the DHE fluorescence levels were significantly lower than the L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS and L-FABP<sup>+/−</sup> AT1a<sup>+/+</sup> RAS mice (P < 0.05). The DHE fluorescence levels in the L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS mice were significantly lower than those in the L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS mice (L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, 10.8 ± 2.8 vs. L-FABP<sup>−/−</sup> AT1a<sup>+/+</sup> RAS, 28.7 ± 6.4%, P < 0.05; Fig. 9).

**Study 2: Change of Renal hL-FABP Expression with Decrease in AT1<sub>a</sub> Expression**

**Dynamics of human L-FABP expression in the kidney.** The mRNA levels of renal hL-FABP (Fig. 10A) in L-FABP<sup>−/−</sup> AT1a<sup>−/−</sup> RAS and L-FABP<sup>+/−</sup> AT1a<sup>−/−</sup> RAS mice were sig-
Fig. 6. The areas in Fig. 5 were assessed quantitatively as described in MATERIALS AND METHODS. Immunohistochemical staining was performed using an antibody against F4/80 in the interstitium (A). The tubulointerstitial damage of histological findings of periodic acid-Schiff (B). Immunohistochemical staining using an antibody against type I collagen (C) and an antibody against type III collagen (D). *$P < 0.05$ vs. the control group on the same day; †$P < 0.05$ vs. the AT1a$^{+/−}$RAS groups on the same day; ‡$P < 0.05$ vs. L-FABP$^{−/−}$ groups on the same day.

Fig. 7. Evaluation of glomerulus. Normal glomerulus and glomerular sclerosis are shown (A). Original magnification, ×200. Glomerular sclerosis score was assessed quantitatively (B), as described in MATERIALS AND METHODS.

Fig. 8. The protein levels of renal hL-FABP (Fig. 10B) in L-FABP$^{+/−}$ or L-FABP$^{−/−}$ control and L-FABP$^{+/−}$/AT1a$^{+/−}$ control mice ($P < 0.05$). In the L-FABP$^{+/−}$/AT1a$^{+/−}$ RAS, L-FABP levels were similar to those in the L-FABP$^{+/−}$/AT1a$^{−/−}$ control. L-FABP levels in the L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS and the L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS mice were significantly lower than those in the L-FABP$^{+/−}$/AT1a$^{+/−}$/RAS mice (L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS, 0.3 ± 0.1 vs. L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS, 1.0 ± 0.1 vs. L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS, 1.4 ± 0.1, $P < 0.05$; values in arbitrary units).

The protein levels of renal hL-FABP (Fig. 10B) in L-FABP$^{+/−}$/AT1a$^{+/−}$/RAS and L-FABP$^{+/−}$/AT1a$^{+/-}$/RAS mice were significantly higher than in the L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS control and L-FABP$^{+/−}$/AT1a$^{+/−}$/RAS control mice ($P < 0.05$). In the L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS, L-FABP protein levels were similar to L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS. L-FABP protein levels decreased along with the decrease in the expression of AT1a, and its levels in the L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS were significantly lower than those in the L-FABP$^{+/−}$/AT1a$^{+/−}$/RAS (L-

FABP$^{+/−}$/AT1a$^{−/−}$/RAS, 3,065 ± 353 vs. L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS, 5,215 ± 542 vs. L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS, 6,556 ± 514 μg/mg protein, $P < 0.05$).

Urinary hL-FABP levels (Fig. 10C) in L-FABP$^{+/−}$/AT1a$^{+/−}$/RAS and L-FABP$^{+/−}$/AT1a$^{+/-}$/RAS mice were significantly higher than in the L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS control and L-FABP$^{+/−}$/AT1a$^{+/−}$/RAS control mice on days 14 and 28 ($P < 0.05$). In the L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS mice, the L-FABP excretion levels were similar to those in the L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS control mice. Its levels in the L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS and the L-FABP$^{+/−}$/AT1a$^{+/-}$/RAS mice were significantly lower than in the L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS mice on days 14 and 28 (L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS, 9.5 ± 2.0 vs. L-FABP$^{+/−}$/AT1a$^{−/−}$/RAS, 144.0 ± 49.5 μg/mg creatinine, $P < 0.05$).

In the L-FABP$^{+/−}$/control mice, its levels on days 14 and 28 were similar to that on day 0 (data not shown).
were assessed quantitatively as described in MATERIALS AND METHODS (P/H11021). AT1a mutant mice, was also found in the glomerulus, in the tubulointerstitial damage in this renal damage model induced by high-dose ANG II infusion and high-salt diet (11) was used to examine the renoprotective effect of renal hL-FABP expression and the decrease in expression of ANG II receptor by using AT1a mutant mice in which the AT1a receptor gene was modified by replacing its gene with the β-galactosidase (LacZ) gene such that the LacZ activity was under transcriptional control of the endogenous AT1a locus (27). Because renal damage was ameliorated along with decrease in mRNA levels of AT1a in the L-FABP−/− mice, this suggests that the action of ANG II via AT1a played an important role in renal damage observed in this model. Activation of AT1a signaling induces ROS production in the proximal tubules, which leads to the tubulointerstitial damage (28). Therefore, renal hL-FABP, which is a scavenger of ROS, is effective in amelioration of the tubulointerstitial damage. This study suggested that the upregulation of expression of renal hL-FABP, which is an antioxidant, together with suppression of AT1a expression, inhibited the production of inflammatory cytokines and attenuated the tubulointerstitial damage in this renal damage model induced by RAS activation.

The current results provide new insights into the dynamics of renal hL-FABP in the reduction of AT1a receptor expression. AT1a receptor was reported to be widely distributed along the nephron including proximal epithelial sites (3). LacZ staining, which corresponds to the expression sites of AT1a in the AT1a mutant mice, was also found in the glomerulus, in the juxtaglomerular apparatus of the renal cortex, and in the proximal tubules of the renal cortex (27). RAS activation and induction of renal injury led to increased expression of renal hL-FABP not only in the proximal tubules of the renal cortex (S1–2) but also in the proximal tubules of the medulla (S3), which was consistent with the area where activation of the AT1a receptor was observed (7). In the current study, upregulation of renal hL-FABP expression by RAS activation and the increase in urinary L-FABP levels was suppressed by reduction of ROS along with reduction of AT1a receptor expression. From these results, it may be inferred that expression of renal hL-FABP in the proximal tubules and urinary excretion of renal hL-FABP changed in response to the amount of ROS produced by activation of ANG II signaling via AT1a. These results appear to mimic the decrease in urinary hL-FABP that was brought about by inhibition of RAS activation due to the activity of angiotensin-converting enzyme inhibitor or ANG II receptor blocker shown in the clinical studies in patients with CKD and hypertension (18, 19, 20).

With regard to the pathophysiological role of renal hL-FABP in the RAS activated renal injury model, upregulation of renal hL-FABP resulted in amelioration of tubulointerstitial damage in addition to renoprotective effects due to the decrease in AT1a signaling. Experimental studies in liver disease have shown that L-FABP is a scavenger of ROS and works as an endogenous antioxidant (25, 29, 31). In this study, upregulation of renal hL-FABP suppressed the production of ROS. A previous study indicated that in the same model, renal hL-
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FABP inhibited renal Ne-(hexanoyl)lysine accumulation, which is an early marker of oxidative stress (7). Because activation of AT1a in the proximal tubules produces ROS, which leads to progression of renal injury (28), the renoprotective effect of renal hL-FABP may be due to its antioxidative activity.

The degree of glomerulosclerosis and urinary albumin level secretion, which reflect the degree of glomerular damage, was not improved in the L-FABP+/−RAS mice. This is because hL-FABP is expressed in the proximal tubules, and so tubulointerstitial damage but not glomerular damage was ameliorated in the L-FABP+/−RAS mice compared with the L-FABP+/−RAS mice.

In the mice with RAS activation, SBP decreased and the tubulointerstitial damage was improved along with reduction of AT1a receptor expression. To examine the influence of decrease in SBP levels on kidney injury, hydralazine was administered to the L-FABP+/−AT1a+/+RAS mice (data not shown). Although SBP decreased to a level comparable to that in the L-FABP+/−RAS mice, protein expression levels of α1-type III collagen in the L-FABP+/−AT1a−/−RAS were significantly lower than those in the L-FABP+/−AT1a−/−RAS mice. Because it was reported that activation of mineralocorticoid receptor played an important role in the renal injury in the RAS activation model (11), it was speculated that AT1a-independent tubulointerstitial fibrosis was induced and was suppressed by the upregulation of renal hL-FABP.

A high dose of ANG II and high-salt diet was reported to lead to progressive augmentation of intrarenal ANG II. Augmentation of ANG II generation was due to increase in tubular angiotensinogen (AGT) synthesis and secretion from the proximal tubules and production of renin and activation of angiotensin-converting enzyme in the collecting ducts, which was accelerated by high-salt intake in addition to ANG II infusion. Because renal AGT mRNA levels and urinary AGT also increase in the AT1a+/+RAS mice in our model (data not shown), this suggested intrarenal RAS activation.

A potential limitation of this study is that we did not show a significant difference of oxidative stress between the kidneys of FABP+/−AT1a+/+RAS and those of FABP+/−AT1a+/−RAS. We could not find a highly precise method that can detect the difference of oxidative stress produced between two groups with mild to moderate tubulointerstitial damage.

RAS activation is a primary aggravating factor for progression of CKD, and inhibition of RAS plays a central role in clinical management of the patients with CKD (12). However, excessive inhibition of RAS is known to lead to acute kidney injury due to decrease in glomerular filtration rate or heart failure due to hyperkalemia in the patients of advanced age or the patients with diabetes (22, 33). Therefore, new treatments for renoprotection apart from inhibition of RAS are needed for CKD. The results of this study suggest that agents that upregulate renal hL-FABP expression may be useful as effective medicines for preventing the progression of CKD together with inhibitors of RAS.

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DISCLOSURES

T. Sugaya is the senior director and senior scientist of CMIC (Tokyo, Japan), which produced the kits for L-FABP analysis. No other potential conflicts of interest relevant to this article are reported.

AUTHOR CONTRIBUTIONS


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