Human liver-type fatty acid–binding protein protects against tubulointerstitial injury in aldosterone-induced renal injury

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Ichikawa D, Kamijo-Ikemori A, Sugaya T, Shibagaki Y, Yasuda T, Hoshino S, Katayama K, Igarashi-Migitaka J, Hirata K, Kimura K. Human liver-type fatty acid–binding protein (hL-FABP) expressed in proximal tubules in aldosterone (Aldo)-induced renal injury, hL-FABP chromosomal transgenic (Tg) and wild-type (WT) mice received systemic Aldo infusions (Tg-Aldo and WT-Aldo, respectively) were given 1% NaCl water for 28 days. In this model, elevation of systolic blood pressure, monocyte chemoattractant protein-1 expression, macrophage infiltration in the interstitium, tubulointerstitial damage, and depositions of type I and III collagens were observed. Elevation of systolic blood pressure did not differ in WT-Aldo vs. Tg-Aldo animals, however, renal injury was suppressed in Tg-Aldo compared with WT-Aldo mice. Dihydroethidium fluorescence was used to evaluate reactive oxidative stress, which was suppressed in Tg-Aldo compared with WT-Aldo mice. Gene expression of angiotensinogen in the kidney was upregulated, and excretion of urinary angiotensinogen was increased in WT-Aldo mice. This exacerbation was suppressed in Tg-Aldo mice. In conclusion, hL-FABP ameliorated the tubulointerstitial damage in Aldo-induced renal injury via reducing oxidative stress and suppressing activation of the intrarenal renin-angiotensin system.

MATERIALS AND METHODS

SALT PREFERENCE, OVEATURING, and obesity associated with the advance of civilization have resulted in excessive activation of the renin-angiotensin-aldosterone system (RAAS) (4), leading to an increase in the number of patients with chronic kidney damage (CKD) due to hypertension. Tubulointerstitial damage is more strongly associated with the progression of kidney disease than the degree of glomerular injury (25), and inappropriate elevation of components produced by activation of the RAAS, such as angiotensin II (Ang II) or aldosterone (Aldo), cause tubulointerstitial damage that can increase in patients with end-stage renal failure (15). Therefore, inhibitors of these components, which include angiotensin-converting enzyme inhibitor (2), Ang II receptor blocker (16), and mineralocorticoid receptor (MR) blocker (21, 26, 32), play a central role in the treatment of CKD.

Liver-type fatty acid–binding protein (L-FABP) is expressed in human renal proximal tubules (18). L-FABP is an effective endogenous antioxidant during oxidative stress generated in pathophysiological conditions (28, 30, 34). Because L-FABP is not expressed in mouse kidneys (27), we generated chromosomal transgenic (Tg) mice in which human L-FABP (hL-FABP) was expressed in the proximal tubules of the cortex by microinjection of hL-FABP genomic DNA, including its promoter region, to evaluate the pathophysiological role of hL-FABP (8). The distribution of hL-FABP expression in the Tg mice was restricted to the kidney, liver, and intestine (8). Animal studies of kidney disease showed that hL-FABP gene expression in the kidney was upregulated by various aggravating factors of kidney disease such as urinary protein overload (8), tubular ischemia (33), tubular stretch (10), hyperglycemia (11), and toxins (19, 35). Furthermore, hL-FABP expression attenuates tubulointerstitial damage by reducing oxidative stress. Moreover, the mechanism of its antioxidant activity depends on the inactivation of free radicals by methionine and cysteine amino acids involved in hL-FABP (34). Some agents that upregulate gene expression of hL-FABP could become new therapeutic targets in preventing the progression of various kidney diseases due to different etiologies.

In studying the role of hL-FABP in kidney injuries due to RAAS activation, we (6, 7) and others (24) recently revealed that hL-FABP reduced oxidative stress and attenuated the tubulointerstitial injury in an Ang II infusion model. In kidney injury due to RAAS activation, the end product of the RAAS, Aldo, is also an important aggravating factor in the progression of kidney injury (1). Aldosterone affects the glomerulus, proximal tubules, distal tubules, and collecting ducts via its receptor, the MR, and it provokes both glomerular injury and tubulointerstitial injury (12). Reactive oxygen species (ROS) produced by excessive Aldo mediate the kidney injuries, and these are attenuated by antioxidants (23). Another investigation found that Aldo activated the intrarenal renin-angiotensin system (RAS) through positive feedback reactions in a systemic Aldo infusion model, and that its activation led to kidney injury (3). Because ROS stimulated the activation of the intrarenal RAS (5, 24, 36), we hypothesized that hL-FABP with antioxidant activity would inhibit the activation of the intrarenal RAS due to Aldo and ameliorate kidney injury. Thus the aim of this study is to demonstrate the pathophysiological significance of hL-FABP in a systemic Aldo infusion model.

Animals. Studies were conducted in accordance with the St. Marianna University School of Medicine Institutional Guide for Animal Experiments and the Guide for the Care and Use of Laboratory
**Animals** (National Academies Press, Washington, DC). Because hL-FABP is not expressed in mouse kidney, hL-FABP Tg mice were generated as described previously (patent WO0073791; World Intellectual Property Organization, Geneva, Switzerland) (8). Male hL-FABP Tg mice (n = 18, 8–10 wk old, body wt 27.5 ± 0.3 g) on a C57/BL6 background and wild-type (WT) mice (n = 18, body wt 26.8 ± 0.4 g) were purchased from Japan SLC (Shizuoka, Japan).

**Model of Aldo-induced renal damage.** Both Tg and WT mice were divided into two groups. The Aldo group (Tg-Aldo n = 11, WT-Aldo n = 11) received a systemic Aldo infusion (A9477; Sigma-Aldrich, 0.125 μg·kg⁻¹·min⁻¹) using an osmotic minipump (Alzet 1004; Durect) for 28 days. Aldo was dissolved in polyethylene glycol (PEG 300; Sigma) and was infused via an implanted osmotic minipump into the subcutaneous space of mice anesthetized with isoflurane. The control group (Tg-control n = 7, WT-control n = 7) was given vehicle only. All mice were provided drinking water containing 1% NaCl during the experimental period. 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 containing 1% NaCl. Mice kidneys and serum were collected on day 28 for various analyses.

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

**Serum and urinary biochemistry.** Serum and urinary creatinine were measured by an enzymatic method (Nescoat VL II CRE; Alfresa Pharma) (7). Serum potassium and sodium were measured by an electrode method, and serum Aldo was measured by a radioimmunoassay provided by the clinical laboratory testing services of SRL. Urinary parameters are reported as ratios relative to urinary creatinine levels. Mouse albuminuria was determined using the Albuwell assay (Exocell). Urinary hL-FABP was measured using a two-step sandwich ELISA procedure (hL-FABP ELISA kit; CMIC, Tokyo, Japan) (8). Mouse angiotensinogen (AGT) in urine collected on day 28 was measured using an AGT ELISA kit (IBL, Gunma, Japan). Urinary lipid peroxidation (LPO) product levels on day 28 were measured using the thioarbituric acid method (Oxiselect TBARS assay kit; Cell Bioslachts).

**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 for immunohistochemistry. Tubulointerstitial injury was categorized as tubular dilation with epithelial atrophy and extracellular matrix accumulation. Under magnification (×200), 10 nonoverlapping fields from the entire cortical areas were selected, and the areas of tubulointerstitial injury were measured using an image analyzer (Winroof version 6.1; Mitani, Japan). Each degree of tubulointerstitial injury was evaluated as ratio relative to the entire cortical area (7). For glomerulosclerosis quantitation, the grade of sclerosis in each glomerulus stained with periodic acid Schiff was defined as described previously (7).

**Immunohistochemical analysis.** Tissues fixed in methyl Carnoy solution were embedded in paraffin. An indirect immunoperoxidase method described previously (19, 35) was used to identify the antigens. Macrophages were identified using the rat monoclonal antibody F4/80 (BMA Biomedicals, Augst, Switzerland), and type I and type III collagens were identified using 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 ROS production.** To evaluate ROS production of the kidneys in situ, unfixed frozen cross-sections from the kidney were stained with 10 μM dihydroethidium (DHE) (Invitrogen) for 30 min in a dark, humidified chamber at 37°C. ROS generation was labeled with red fluorescence and visualized by fluorescence microscopy (6, 17). The DHE fluorescence intensities of kidney cross-

![Fig. 1. Time-related changes in systolic blood pressure (A) and urinary albumin levels (B). Wild-type aldosterone (WT-Aldo) n = 11 mice, wild-type control (WT-control) n = 7, transgenic-aldosterone (Tg-Aldo) n = 11, transgenic control (Tg-control) n = 7. Time course of *P < 0.05 vs. the control group on the same day; †P < 0.05 vs. the same group on day 0; ‡P < 0.05 vs. the same group on day 14. NS, not significant.](http://ajprenal.physiology.org/)

<table>
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<tr>
<th>Parameter</th>
<th>WT-Control Mice</th>
<th>WT-Aldo Mice</th>
<th>Tg-Control Mice</th>
<th>Tg-Aldo Mice</th>
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<td>Creatinine, mg/dl</td>
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<td>Sodium, meq/liter</td>
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<td>Potassium, meq/liter</td>
<td>4.6 ± 0.3</td>
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<td>Aldosterone, pg/ml</td>
<td>119.2 ± 17.5</td>
<td>2,936.0 ± 88.0*</td>
<td>70.2 ± 19.1</td>
<td>2,697.9 ± 339.9*</td>
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Data are means ± SE. Aldo, aldosterone; Tg, transgenic; WT, wild-type. *P < 0.05 vs. the control group on the same day.
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 chemotactrant protein-1 and hL-FABP by ELISA. Proteins were extracted from frozen kidneys and protein concentration was measured as described previously (7). The inflammatory cytokine monocyte chemotactrant protein-1 (MCP-1) and hL-FABP were measured by ELISA (R&D Systems for MCP-1; CMIC for hL-FABP) (7). The concentrations of MCP-1 and hL-FABP were corrected for total protein concentration.

Real-time quantitative polymerase chain reaction analysis. Total RNA was extracted and reverse transcribed as described previously (19, 35). TaqMan real-time polymerase chain reaction (PCR) was performed using a StepOnePlus real-time PCR system (Applied Biosystems). Real-time PCR was used to detect the mRNA of hL-FABP, MCP-1, α1 type I collagen, α1 type III collagen, catalase, nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), AGT, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression levels of those mRNAs in each sample were normalized to GAPDH expression levels. ACC, gene expression of angiotensinogen (AGT), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Ex-

RESULTS

Blood pressure in response to Aldo. Systolic blood pressures of Tg-Aldo mice were similar to those of WT-Aldo mice from day 7 to day 28 (Fig. 1A).

Serum and urinary biochemistry. Serum creatinine levels did not differ significantly between the four groups of mice (WT-control, WT-Aldo, Tg-control, and Tg-Aldo) (Table 1). Serum Aldo levels in WT-Aldo and Tg-Aldo mice were significantly higher than they were in control mice (P < 0.05, Table 1).
There were no significant differences in serum Aldo levels in WT-control and Tg-control mice.

Urinary albumin levels did not differ significantly between Tg-Aldo and WT-Aldo mice on day 14 and day 28 (Fig. 1B).

Expression of MCP-1 in the kidney. MCP-1 is an inflammatory cytokine released to the interstitium of kidneys from the proximal tubules. Gene expression levels of MCP-1 in Tg-Aldo mice were significantly lower than in WT-Aldo mice \((P < 0.05, \text{Fig. 2A})\). Protein expression levels of MCP-1 in Tg-Aldo mice were significantly lower than those measured in WT-Aldo mice \((P < 0.05, \text{Fig. 2B})\).

Evaluation of macrophage infiltration. To examine the degree of tubulointerstitial inflammation, we assessed the degree of macrophage infiltration via immunohistochemical analysis using the rat monoclonal antibody, F4/80. Macrophage infiltration into the kidney cortexes of Tg-Aldo mice was significantly less than it was in WT-Aldo mice (Fig. 3, A and F).

Renal histological and morphometric analysis. In Tg-Aldo and WT-Aldo mice, periodic acid Schiff-stained sections revealed tubulointerstitial damage, including dilatation of tubules and degeneration of proximal tubular epithelial cells (Fig. 3B). There was significantly less tubulointerstitial damage of the cortex in Tg-Aldo mice than in WT-Aldo mice \((P < 0.05, \text{Fig. 3G})\). Glomerular sclerosis scores were not significantly different among groups (Fig. 3, E and J).

Immunohistological analysis of type I and type III collagens. To examine the degree of tubulointerstitial fibrosis, we assessed type I and type III collagen expression using immunohistochemical analysis and TaqMan real-time PCR. Deposition levels of type I collagen (Figs. 3C and 3H) and type III collagen (Figs. 3D and 3I) on the kidney cortex in Tg-Aldo mice were significantly lower than in WT-Aldo mice \((P < 0.05)\). Gene expression levels of \(\alpha\)-1 type I collagen (Fig. 2C) and \(\alpha\)-1 type III collagen (Fig. 2D) in the kidneys of Tg-Aldo mice were assessed quantitatively as described in MATERIALS AND METHODS.

Fig. 3. Histological findings. A: immunohistological staining using an antibody against F4/80. B: histological staining with periodic acid Schiff (PAS), also showing tubulointerstitial damage. Immunohistological staining using an antibody against type I collagen (C) and an antibody against type III collagen (D). A, B, C and D were assessed quantitatively as described in MATERIALS AND METHODS. E: glomerulus stained with PAS. Original magnification \(\times 200\). F: level of immunohistological staining using an antibody against F4/80 per field in the interstitium. G: histological PAS staining indicating tubulointerstitial damage. Deposition levels of type I collagen (H) and type III collagen (I). J: glomerular sclerosis score. WT-Aldo \(n = 11\) mice, WT-control \(n = 7\), Tg-Aldo \(n = 11\), Tg-control \(n = 7\). *\(P < 0.05\) vs. the control group on the same day; #\(P < 0.05\) vs. the L-FABP-Tg group on the same day.
mice were significantly lower than in WT-Aldo mice (P < 0.05).

Evaluation of oxidative stress. Oxidative stress was evaluated using various parameters in the present model. Catalase is the principal antioxidant enzymes in the kidney, Nrf2 has an important function in protection against oxidative stress, and HO-1 is a microsomal enzyme induced by oxidative stress. Gene expression levels of hL-FABP in Tg-Aldo mice were significantly lower than in WT-Aldo mice (P < 0.05).

To determine the influence of hL-FABP on ROS production, DHE staining was performed on kidney sections. DHE fluorescence levels in Tg-Aldo mice were significantly lower than those in WT-Aldo mice (P < 0.05, Fig. 4, D and E). DHE fluorescence levels in Tg-Aldo mice were significantly lower than they were in WT-Aldo mice (P < 0.05, Fig. 4F).

Influence of the MR on hL-FABP and AGT expressions. To examine whether hL-FABP and AGT expression levels are modulated by the MR, Tg-Aldo mice were injected with the MR blocker, spironolactone, for 28 days. Although blood pressure levels in both LSp and HSp groups tended to be lower than those in Tg-Aldo mice on day 14, blood pressures increased to a similar extent in both Sp groups and the Aldo group on day 28 (Fig. 6A). Gene expression of hL-FABP in kidneys of the HSp group tended to be lower than that in the Aldo group (Fig. 6B), and hL-FABP protein expression in kidneys of the HSp group was significantly lower than that in the Aldo group (P < 0.05, Fig. 6C). Urinary hL-FABP levels in the HSp group were significantly lower than those in the Aldo group on day 28 (P < 0.05, Fig. 6D).

Gene expression of AGT in the kidneys of the HSp group was significantly lower than that in the Aldo group (P < 0.05, Fig. 6E) and urinary AGT levels in the HSp group tended to be lower than those in the Aldo group on day 28 (Fig. 6F).

DISCUSSION

This study, which utilized a model of Aldo-induced nephropathy, showed that upregulation of hL-FABP (an antiox-

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**Fig. 4. Evaluation of oxidative stress in the kidney.** A: gene expression of catalase mRNA transcripts. B: gene expression of nuclear factor erythroid 2-related factor 2 (Nrf2) mRNA transcripts. C: gene expression of heme oxygenase-1 (HO-1) mRNA transcripts in the kidney. D: production of reactive oxygen species indicated by dihydroethidium (DHE) fluorescence in the kidney. Original magnification ×200. E: the areas in D were assessed quantitatively using DHE as described in MATERIALS AND METHODS. F: urinary lipid peroxidation (LPO) levels on day 28. WT-Aldo n = 11 mice, WT-control n = 7, Tg-Aldo n = 11, Tg-control n = 7. *P < 0.05 vs. the control group on the same day; #P < 0.05 vs. the L-FABP-Tg group on the same day.

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In the kidney of Tg-control mice on day 28, and medulla (F) filtered through glomeruli and promoted production of renal Ang II (20), it was difficult to demonstrate how AGT is filtered through glomeruli, and the amount of filtered AGT increased only slightly, even in a glomerular injury model with RAS activity. Therefore, increased AGT production in proximal tubules was an important factor in activation of the intrarenal RAS (22). Therefore, ROS produced by stimulation of Aldo in the proximal tubules may activate the intrarenal RAS, and hL-FABP may diminish the tubular damage. The present study showed that the gene expression of renal AGT and urinary AGT levels, which accurately reflect the degree of intrarenal RAS activation (14), were significantly higher in WT-Aldo kidneys than in WT-control kidneys. AGT increases were significantly inhibited in Tg-Aldo kidneys with hL-FABP. From these results, it was considered that Aldo activated the early pathways of the RAS and that hL-FABP, which is an ROS scavenger, attenuated tubulointerstitial damage via reducing the intrarenal RAS activation by ROS.

Does intrarenal activation of the AT1 receptor cause tubulointerstitial damage in Aldo-induced nephropathy? The AT1 receptor in rodents exists as two isoforms, AT1a and AT1b. AT1a plays a major role in the renal actions of Ang II. To resolve this question, we made a systemic Aldo infusion model using homozygous mutant mice in which the AT1a receptor gene was disrupted (AT1a−/−) (29). Tubulointerstitial damage and gene expression of MCP-1 in kidneys of AT1a−/−-Aldo mice were significantly suppressed compared with those in AT1a+/−-Aldo mice (data not shown). Previous studies showed that inhibition of the AT1 receptor ameliorated kidney damage in Aldo-induced nephropathy (3). These results indicated that...
intrarenal activation of the AT1 receptor contributed to tubulointerstitial injury in Aldo-induced nephropathy.

We examined the influence of increased blood pressure on kidney injury. Hydralazine was administered to WT-Aldo mice for 28 days (data not shown). Although systolic blood pressure decreased to a level comparable to that in control mice without Aldo, tubulointerstitial damage was not ameliorated. This could be because the increase in systolic blood pressure was not strongly associated with progression of tubulointerstitial damage observed in WT-Aldo mice.

Aldosterone injected subcutaneously is absorbed into the systemic circulation and acts on the proximal tubules via MR (31). Blood pressure; serum Aldo, sodium, and potassium levels; and gene expression levels of MR in the kidneys of Tg-Aldo and WT-Aldo mice showed no significant differences (data not shown). The degree of glomerular injury and urinary albumin levels in these two groups did not differ. Therefore, it was speculated that the amount of Aldo loaded on the kidneys was equivalent in the two groups.

The results of research with a blocker of MR, spironolactone, suggested that the upregulation of hL-FABP and AGT expression depended on the degree of MR activity. Because activation of MR generates intracellular ROS, the ROS derived from activation of the MR may be associated with upregulation of hL-FABP and AGT expression. Therefore, we concluded that a blocker of MR inhibited the generation of ROS, which led to a decrease in the expression levels of hL-FABP and AGT upregulated via the activation of MR in the proximal tubules.

Major consequences of RAAS activation are widely known to contribute significantly to kidney damage. In addition to the protection provided by hL-FABP in Ang II-induced nephropathy, this study demonstrates that hL-FABP also attenuates tubulointerstitial damage in Aldo-induced nephropathy by reducing oxidative stress. Agents that upregulated the expression of hL-FABP might be a new strategy for preventing the progression of tubulointerstitial damage in kidney injury due to RAAS activation.

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DISCLOSURES

T. Sugaya is the Director and Senior Scientist of CMIC Holdings Co. Ltd., the company that produced the kits for L-FABP analysis. None of the other authors have conflicts of interest or financial disclosures of any relevance to the present study.
AUTHOR CONTRIBUTIONS


REFERENCES