Kidney

Amelioration of Angiotensin II–Induced Salt-Sensitive Hypertension by Liver-Type Fatty Acid–Binding Protein in Proximal Tubules

Ken Osaki, Yusuke Suzuki, Takeshi Sugaya, Chiaki Tanifuji, Akira Nishiyama, Satoshi Horikoshi, Yasuhiko Tomino

Abstract—Inappropriate activation of the intrarenal renin–angiotensin system induces generation of reactive oxygen species and tubulointerstitial inflammation, which contribute to salt-sensitive hypertension (SSHT). Liver-type fatty acid–binding protein is expressed in proximal tubules in humans, but not in rodents, and may play an endogenous antioxidative role. The objective of the present study was to examine the antioxidative effect of liver-type fatty acid–binding protein on post–angiotensin II SSHT model in transgenic mice with selective overexpression of human liver-type fatty acid–binding protein in the proximal tubules. The transgenic mice showed marked protection against angiotensin II–induced SSHT. Overexpression of tubular liver-type fatty acid–binding protein prevented intrarenal T-cell infiltration and also reduced reactive oxygen species generation, intrarenal renin–angiotensin system activation, and monocyte chemotactic protein-1 expression. We also performed an in vitro study using the murine proximal tubular cell lines with or without recombinant liver-type fatty acid–binding protein and murine proximal tubular cell lines transfected with human liver-type fatty acid–binding protein, and found that gene transfection of liver-type fatty acid–binding protein and, in part, recombinant liver-type fatty acid–binding protein administration had significantly attenuated angiotensin II–induced reactive oxygen species generation and the expression of angiotensinogen and monocyte chemotactic protein-1 in murine proximal tubular cell lines. These findings indicated that liver-type fatty acid–binding protein in the proximal tubules may protect against angiotensin II–induced SSHT by attenuating activation of the intrarenal renin–angiotensin system and reducing oxidative stress and tubulointerstitial inflammation. Present data suggest that liver-type fatty acid–binding protein in the proximal tubules may be a novel therapeutic target for SSHT. (Hypertension. 2013;62:712-718.) • Online Data Supplement

Key Words: kidney tubules, proximal ▪ liver fatty acid–binding protein ▪ oxidative stress ▪ renin-angiotensin system

The renin–angiotensin system (RAS) is a major mediator of hypertension and a key target in the treatment of this disease. It was recently demonstrated that angiotensin II (AngII) increases T-cell infiltration into the tubulointerstitium, with subsequent induction of T-cell activation and production of proinflammatory cytokines. These changes are known to play an important role in the development of salt-sensitive hypertension (SSHT).1-3

The major components of the RAS are expressed in the renal proximal tubular cells (PTCs); therefore, it has been postulated that PTC-derived AngII may be more important than circulatory-derived AngII for inducing SSHT.4 AngII induces the production of reactive oxygen species (ROS) in PTCs mainly through the activation of nicotinamide adenine dinucleotide phosphate oxidase. AngII-induced ROS are also potent inter- and intracellular secondary messengers that mediate various signaling pathways involved in the intrarenal inflammation and hypertension. Therefore, antioxidant treatment may ameliorate AngII-induced hypertension5 and AngII-related tubulointerstitial injury in IgA nephropathy.6

Liver-type fatty acid–binding protein (L-FABP) is a 14-kDa protein found in the cytoplasm of human PTCs.7 Fatty acids are bound to L-FABP and transported to the mitochondria or peroxisomes, where they are β-oxidized. This reaction plays a role in fatty acid homeostasis.8 L-FABP also has a high affinity and capacity to bind to long-chain fatty acid oxidation products and, therefore, acts as effective endogenous antioxidant.9,10 Because renal L-FABP is not expressed in the kidneys of rodents,11 it is necessary to generate human L-FABP chromosomal transgenic (hL-FABPTg) mice to examine the pathophysiological role of renal L-FABP in human kidney diseases experimentally. The pathological significance of hL-FABP has been determined in many experimental models in which oxidative stress might contribute to the progression of
tubulointerstitial injury, such as protein overload nephropathy, diabetic nephropathy, and unilateral ureteral obstruction. The expression of renal hL-FABP is upregulated in the pathological conditions in these mice, with tubulointerstitial inflammation and fibrosis being markedly attenuated compared with wild-type (WT) mice. However, the pathophysiological significance of tubular hL-FABP in local RAS activation and subsequent SSHT remains to be determined. The present study analyzed AngII-induced SSHT in hL-FABP Tg mice and investigated the underlying mechanism in PTCs with or without hL-FABP transfection or treatment of recombinant L-FABP peptides (rhL-FABP).

Methods
A detailed description of all methods is presented in the online-only Data Supplement.

Results
AngII-Induced SSHT Was Prevented in hL-FABP Tg Mice
All groups had the same level of systolic blood pressure (SBP; 102–112 mm Hg) at the beginning of the study (Figure 1). AngII infusion in the WT mice induced a progressive increase in SBP (159.1±13.7 and 177.2±10.7 mm Hg at weeks 2 and 4, respectively; *P<0.001 versus WT before and AngII hL-FABP Tg at week 4; Figure 1). However, AngII infusion in hL-FABP Tg mice induced a small increase in SBP reaching a peak at week 2 (150.5±19.6 and 156.6±19.8 mm Hg at weeks 2 and 4, respectively; *P<0.001 versus hL-FABP Tg before; Figure 1). Withdrawal of AngII infusion was followed by a sharp fall in SBP to near-normal levels in both strains of the mice (Figure 1). However, subsequent administration of high salt diet (HSD) to AngII-infused WT mice caused a re-elevation of SBP (121.5±4.9 and 119.7±5.5 mm Hg at weeks 1 and 4 after HSD, respectively; *P<0.001 versus AngII hL-FABP Tg). These findings indicated that the mice had acquired SSHT (Figure 1). In marked contrast, SBP levels in AngII-infused hL-FABP Tg mice remained largely unchanged during the 4-week HSD for 4 weeks indicating that these mice had not developed SSHT.

Enhancement of Intrarenal Angiotensinogen Expression Was Prevented in hL-FABP Tg Mice
To evaluate activation of the intrarenal RAS, we measured the expression of renal angiotensinogen (AGT) because it has been shown to have a significant relationship with intrarenal AngII and is, therefore, a useful index of its activity.

Figure 2A and 2B shows the effects of AngII infusion on AGT protein. Immunohistochemical analysis (Figure 2A) demonstrated that AGT was expressed mainly in the proximal tubules and was enhanced only in WT mice during AngII infusion with expression being sustained during HSD administration (*P<0.05). AngII infusion in WT mice also caused a significant increase in urinary AGT (25.4±10 µg/gCr at 4 weeks; n=4; versus sham-treated WT; *P<0.05). Urinary AGT levels were further increased by the HSD (34.6±9.8 µg/gCr at 9 weeks; n=4; versus sham-treated WT; *P<0.05; Figure 2B). In contrast, AngII infusion in hL-FABP Tg mice did not increase urinary AGT during the study (10.6±5.5 and 12.0±2.0 µg/gCr at weeks 4 and 9, respectively; n=4; Figure 2B). In addition, AngII infusion and HSD enhanced urinary L-FABP excretion in Tg mice (Figure 2C).

Oxidative Stress Was Significantly Attenuated in hL-FABP Tg Mice
Heme oxygenase-1 is considered to be one of the most sensitive indicators of cellular oxidative stress. The other indicators include 4-hydroxy-2-nonenal, a major aldehyde product of lipid peroxidation, and 8-hydroxy-2'-deoxyguanosine, an oxidized nucleoside of DNA. Renal expression of heme oxygenase-1 (Figure 3A) and 4-hydroxy-2-nonenal (Figure 3B) at weeks 4 and 9 and urinary 8-hydroxy-2'-deoxyguanosine level at week 4 (Figure 3C) was significantly lower in hL-FABP Tg mice than in WT mice (*P<0.05). This suggested that the tubular L-FABP attenuates AngII-induced oxidative stress.

Renal Inflammation Was Attenuated in hL-FABP Tg Mice
To assess the effects of L-FABP on intrarenal inflammatory responses, we performed an immunohistochemical analysis of monocyte chemotactic protein (MCP)-1, a key inflammatory cytokine. Renal expression of MCP-1 at week 4 was significantly lower in hL-FABP Tg mice than in WT mice (*P<0.05; Figure 4). Evaluation of CD3+ T-cell infiltration into the kidneys showed that the cells were located particularly around perivascular regions (Figure 5A–5D) with 24% of the vessels from kidneys of AngII-infused WT mice showing moderate to severe perivascular T-cell infiltration (*P<0.05; Figure 5B). In contrast, T-cell infiltration in hL-FABP Tg mice after AngII infusion was suppressed significantly (*P<0.05; Figure 5B). F4/80+ macrophage infiltration was also significantly attenuated in hL-FABP Tg mice with AngII infusion (*P<0.05; Figure 5C).

AngII-Induced ROS Generation and Inflammatory Mediators Were Significantly Attenuated by L-FABP Transfection (mProx-L) or Administration of rhL-FABP in the Murine PTC Lines
To further determine whether L-FABP prevents ROS generation in the PTCs, intracellular O2− levels were measured.
using dihydroethidium. AngII-stimulation resulted in not only L-FABP in murine PTC lines (mProx; Figure 6A) but also a significant increase of intracellular O$_2^-$ levels in mProx at 3 hours with the expression level being considerably higher than that in both mProx with rL-FABP and mProx-L ($P < 0.05$; Figure 6B).

We also investigated whether hL-FABP in PTC attenuates production of AngII-induced inflammatory mediators, such as AGT and MCP-1, as seen in the SSHT models. To check the effect of endogenous and exogenous hL-FABP, we used mProx-L and rhL-FABP. AngII-stimulation ($10^{-7}$ mol/L) resulted in a significant increase in mRNA and protein expressions of both AGT and MCP-1 in mProx at 12 hours. Transfection of hL-FABP in mProx significantly attenuated both mRNA and protein expressions of AGT (Figure 6C). This transfection also significantly attenuated MCP-1 expression (Figure 6D). Although rhL-FABP did not change AGT expression (Figure 6C), mProx with rhL-FABP showed significant attenuation of MCP-1 mRNA expression (Figure 6D).
In human kidneys, L-FABP is expressed predominantly in the PTCs, but not in glomeruli, distal tubules, loop of Henle, or collecting ducts.12 The protein acts as an endogenous antioxidant, and animal experiments have demonstrated a renoprotective role of L-FABP in the tubulointerstitial diseases as a consequence of its antioxidant function.12–14 We recently reported that L-FABP also attenuated chronic and acute glomerular diseases, such as IgA nephropathy and anti-glomerular basement membrane antibody-mediated glomerulonephritis.15,16 Furthermore, recent report demonstrated that L-FABP in PTCs attenuates activation of the intrarenal RAS under AngII infusion.17 The present study provides direct evidence that L-FABP attenuates activation of the intrarenal RAS and AngII-induced SSHT in the PTCs and suggests that oxidative stress may contribute to the pathogenesis and progression of these changes.

Lombardi et al18 reported that short-term (2 weeks) exposure to AngII in rats was followed by the development of SSHT in association with focal tubulointerstitial injury without apparent glomerular damage. Other groups have also demonstrated that intrarenal RAS activation and renal T-cell infiltration after short-term exposure to AngII are linked to SSHT.3,19 AGT is expressed mainly in the renal PTCs and is enhanced during infusion of AngII.4 It was reported that AngII-induced AGT production in PTCs contributes to a further increase in intrarenal AngII levels via activation of the angiotensin II type 1 (AT1) receptor.4 However, AngII increases expression of inflammatory mediators, such as renal MCP-1, mainly via the AT1 receptor.20 Upregulation of renal AngII and MCP-1 induces infiltration and subsequent activation of T cells in the kidneys with the T cells causing further AngII and ROS generation in the tubulointerstitium.21 The previous reports indicated that initial T-cell infiltration caused by AngII may play an important role in the pathogenesis of SSHT.3,18,19 In this study, we demonstrated that the overexpression of L-FABP in PTCs attenuated AngII-induced SSHT associated with a marked decrease in intrarenal AGT expression and urinary AGT excretion among Tg

Figure 4. Renal expression of monocyte chemotactic protein (MCP-1). Renal MCP-1 expression was attenuated significantly in the angiotensin II (AngII) group of human liver-type fatty acid–binding protein chromosomal transgenic (L-FABPTg) mice. Semiquantification of MCP-1 immunostaining in kidney is shown in the following: a, control group of wild-type (WT) mice; b, AngII group of WT mice on AngII infusion; c, AngII group of WT mice on a substantial high-salt diet (HSD); d, Control group of human L-FABPTg (hL-FABPTg) mice; e, AngII group of hL-FABPTg mice on AngII infusion; and f, AngII group of hL-FABPTg mice on substantial HSD. *P<0.05 vs control and AngII group of hL-FABPTg mice. W indicates weeks.

Figure 5. T-cell and macrophage infiltration into the kidney after angiotensin II (AngII) infusion. The T cells and macrophages in the kidney are stained brown. T-cell infiltrates after AngII infusion were detected mainly in the perivascular area in wild-type (WT) mice (A). Perivascular T-cell infiltration was reduced significantly in human liver-type fatty acid–binding protein chromosomal transgenic (L-FABPTg) mice (B). Macrophage infiltration to tubulointerstitial was also significantly attenuated in hL-FABPTg mice with AngII (C). a, Control group of WT mice; b, AngII group of WT mice on substantial high salt diet (HSD); c, Control group of human L-FABPTg (hL-FABPTg) mice; d, AngII group of hL-FABPTg mice on substantial HSD. *P<0.05.
mice at the end of AngII infusion. This suggested that AngII-induced AGT production in the PTCs and development of intrarenal AngII activation may be prevented by the action of L-FABP. The present in vivo and in vitro findings suggest that the effect of tubular L-FABP blocking AngII-induced AGT and MCP-1 overexpression may thus inhibit T-cell infiltration and subsequent further intrarenal RAS activation, which may contribute to preconditioning for SSHT.

Intrarenal oxidative stress may play an important pathological role in the development of SSHT. AngII is a potent activator of tubular nicotinamide adenine dinucleotide phosphate oxidase via the AT1 receptor and, therefore, augments production of ROS, which act as potent inter- and intracellular secondary messengers. Kobori et al. demonstrated that AngII increases AGT expression via ROS-mediated nuclear factor-kB signaling, which could be blocked by antioxidants. MCP-1 expression is also enhanced by AngII-induced nuclear factor-kB signaling. However, local elevation in AngII and ROS caused by infiltrated leukocytes, including T cells, also enhances renal Na+ absorption by direct stimulation of sodium-potassium adenosine triphosphatase and sodium/hydrogen exchanger-3 activity in the renal proximal tubules. It has also been shown that mice lacked T and B cells (recombination activating gene [RAG]-1−/−); mice given T cells from WT C57BL/6 mice develop AngII-induced hypertension, whereas RAG-1−/− mice given T cells from p47 phox−/− mice had attenuated AngII-induced hypertension. These findings indicate that the generation of ROS by PTCs and T cells via nicotinamide adenine dinucleotide phosphate oxidase is a requisite for the development of intrarenal RAS and AngII-induced SSHT. Indeed, the antioxidative action of catalase on PTCs has been reported to attenuate AngII-induced hypertension.

AngII increases generation of hydrophobic ROS, such as superoxide anion (O2−) and hydrogen peroxide via the action of nicotinamide adenine dinucleotide phosphate oxidase. The antioxidant activity of L-FABP is thought to result from the inactivation of free radicals by methionine and cysteine amino acids in L-FABP and also by exposure of the L-FABP–binding
site to lipid peroxides, indicating that L-FABP has both hydrophilic and lipophilic antioxidant effects. The recent study showed that L-FABP has greater hydrophilic antioxidative activity than lipophilic antioxidative activity. These findings indicate that the hydrophilic antioxidative effect of tubular L-FABP in PTCs attenuates AngII-induced SSHT via prevention of AngII-induced ROS generation. Indeed, the present in vitro study demonstrated that AngII-induced intracellular O$_2^-$ generation was significantly attenuated in mProx-L. Interestingly, even rHL-FABP also attenuated the O$_2^-$ generation in mProx as same as mProx-L, suggesting that not only endogenous but also exogenous L-FABP may have hydrophilic antioxidative effect under AngII stimulation. However, a significant attenuation in AGT and MCP-1 expression was observed in mProx-L, whereas rHL-FABP significantly suppressed only MCP-1 mRNA expression. It seems that the present condition of rHL-FABP may be effective for short-term action of AngII, such as O$_2^-$ generation, but not for long-term action of AngII, such as AGT and MCP-1 expression.

### Perspectives

The present study showed that L-FABP in PTC may protect against AngII-induced SSHT by reducing oxidative stress and tubulointerstitial inflammation and subsequent further intrarenal RAS activation. These findings indicate that oxidative stress of the proximal tubules may be a critical inducer for SSHT. Therefore, L-FABP in the renal PTCs may be a novel therapeutic target for hypertension. Peroxisome proliferator-activated receptor agonist enhancing tubular L-FABP or even the recombinant L-FABP would be one of the candidates in this strategy.

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### Disclosures

None.

### References


**Novelty and Significance**

**What Is New?**
- This is the first report to demonstrate a protective role of tubular liver-type fatty acid–binding protein in salt-sensitive hypertension on the basis of intrarenal renin–angiotensin system activation.

**What Is Relevant?**
- Liver-type fatty acid–binding protein in proximal tubular cells is a novel therapeutic target for hypertension.

**Summary**
Liver-type fatty acid–binding protein in proximal tubules protected against angiotensin II–induced salt-sensitive hypertension by reducing oxidative stress and tubulointerstitial inflammation and subsequent intrarenal renin–angiotensin system activation.
Amelioration of Angiotensin II–Induced Salt-Sensitive Hypertension by Liver-Type Fatty Acid–Binding Protein in Proximal Tubules

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ONLINE SUPPLEMENT

Amelioration of AngiotensinII-induced salt-sensitive hypertension by L-FABP in proximal tubules

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A running title: Amelioration of hypertension by tubular L-FABP

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Supplemental Methods

Animals
Since Liver-type fatty-acid binding protein (L-FABP) is not expressed in murine kidneys, we generated hL-FABP chromosomal transgenic mice as described previously (patent no. WO0073791). Briefly, human L-FABP chromosomal transgenic (hL-FABPTg) mice were backcrossed for more than six generations with C57BL/6 mice to obtain homozygous mutant mice with an inbred background.

The mice were housed in the animal facilities of the Juntendo University Faculty of Medicine, with free access to food and water. Eight- to 12-week-old female hL-FABPTg mice (n = 9; 18–25 g) and wild-type (WT) littermates with a C57/BL6 background (n = 9; 17–23 g) were used in this study. The experimental protocol was approved by the Ethics Committee for Animal Experimentation of Juntendo University Faculty of Medicine.

Experimental Protocol
We divided the WT mice and hL-FABPTg mice into two groups. The angiotensin II (AngII) group received AngII (Sigma, St. Louis, MO, USA) (500 ng/kg/min) for 4 weeks using subcutaneous osmotic minipumps (Alzet model 1004, Alza Corp., Palo Alto, CA, USA). At week 4, the AngII infusion was stopped by removing of the minipumps. The mice were fed a normal salt diet (0.4% NaCl) during the AngII infusion period and 1 week after. The mice were then placed on a high salt diet (HSD) (Oriental Yeast Co., 6% NaCl) for an additional 4 weeks. The control group was sham operated and received a normal salt diet during the AngII infusion period and an HSD during the HSD periods. The minipump was implanted into the subcutaneous of mice anesthetized initially with sodium pentobarbital (40 mg/kg IP) and maintained on 2–3% isoflurane.

Physiological assessments
Systolic blood pressure (SBP) was measured in conscious, restrained mice by tail-cuff plethysmography (BP-98, Softron, Tokyo, Japan). The mice were conditioned to the procedure twice before basal BP was measured. Urine was collected for 24 h using metabolic cages (mouse metabolic cage. CLEA, Shizuoka, Japan). Urinary albumin and creatinine concentrations were measured by immunoassay (DCA 2000 system. Bayer Diagnostics, Elkhart, Ind., USA) and expressed as the urinary albumin/creatinine ratio. Urinary angiotensinogen (AGT) concentration was measured using mouse AGT ELISA (code no. 27413, IBL, Gumma, Japan) and the urinary AGT/creatinine ratio then calculated. Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentration was measured using a high sensitive ELISA kit (JaICA, Shizuoka, Japan) and the results expressed as the urinary 8-OHdG/creatinine ratio. Urinary L-FABP was measured by a sandwich ELISA kit using a specific monoclonal antibody to human L-FABP (CMIC Co. Ltd, Tokyo, Japan). Urinary hL-FABP (nanograms per milliliter) was expressed as the urinary hL-FABP/creatinine ratio.

Isolation of RNA, reverse transcription and quantitative RT-PCR
Total RNA was isolated using TRIZOL reagent (Life Technologies Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions. One-microgram aliquots of total RNA
were reverse-transcribed using Random Decamers (Ambion, Austin, TX, USA) and reverse transcriptase, M-MLV (Invitrogen Life Technologies, Carlsbad, CA, USA). The products were then subjected to RT-PCR using an Applied Biosystems 7500 Real Time PCR System with SYBR Green Master Mix (Applied Biosystems, Tokyo, Japan) and specific primers. The gene expression of the target sequence was normalized to that of 18S ribosomal RNA (18S rRNA). The forward and reverse primers used for each molecule were as follows: 5’-AAAGCA GGAGGGAGGAACAG-3’ and 5’-GTAGATGGCGAAGGAGAAGG-3’ for mouse AGT, 5’-AAACGTGCAGAATGGGAAG-3’ and 5’-TCTTCCCTCTTATGCTCC-3’ for human L-FABP, and 5’-TCCCAATGAGTGGCTGAG-3’ and 5’-CCTCTCTCTGAGGCTTGGA-3’ for mouse monocyte chemotactic protein (MCP)-1.

**Histological analyses**
The mice were sacrificed at the end of AngII infusion and after 9 weeks on the HSD. Their kidneys were perfused with ice-cold normal saline and 4% paraformaldehyde. One kidney was then fixed in 4% paraformaldehyde overnight. For immunohistochemical analysis, the paraffin sections were stained with the following antibodies: rabbit anti-mouse haem oxygenase-1 (HO-1) (Abcam, CA, USA), goat anti-mouse MCP-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-mouse angiotensinogen (code no. 99666, IBL, Gumma, Japan) and mouse anti-mouse 4-hydroxy-2-nonenal (4-HNE) (JaICA, Shizuoka, Japan). For each section, five fields were selected randomly, and antibody protein production measured by immunohistochemical-positive staining using the KS400 version 3.0 image analysis system (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). To assess T lymphocyte and macrophage infiltration, the paraffin sections were stained with rabbit anti-mouse CD3 antibody (Abcam, CA, USA) and rabbit anti-mouse F4/80 antibody (Abcam, CA, USA). For each section, 20 randomly-selected fields were scored in a blind fashion for T cell infiltration (3 or more T cells in field). To quantify the extent of vascular inflammation, the vessels were identified on the sections, and the severity of perivascular T cell infiltrates was scored using a previously established method. The vessels were assigned to quartiles as either normal, no T cells present; minimal, 1–4 T cells in infiltrates; moderate, 5–10 T cells in infiltrates; and severe, >10 T cells in infiltrates. The F4/80 positive cells were counted in 10 randomly chosen fields (×400) within the same section of the renal cortex from an individual mouse.

**Cell culture**
mProx, a stable C57BL/6 mouse proximal tubular cell line, was obtained as described previously. Since mProx does not express L-FABP, we transfected hL-FABP including the promoter region into mProx cells using FuGene 6 (Roche) with the ‘resultant’ mProx showing stable expression of hL-FABP being named mProx-L. Both mProx and mProx-L were maintained in the Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS) at 37°C in a 5% CO₂ environment. The cultured cells were grown to confluence in all the experiments and the growth then arrested prior to the commencement of the experiments by culture for 16 h in DMEM medium containing 0.5% FCS.
Preparation of recombinant L-FABP peptides (rhL-FABP)
The complete full length human L-FABP expressed vector has been constructed and introduced into a genus of Gram-positive bacteria. For the expression of rhL-FABP, the jar fermentation was performed and the cell free broth (CFB) was prepared by centrifugation and micro-filtration (pore size: 0.22µm) processes. The expressed protein was purified from CFB after 80% ethanol precipitation and reconstituted with 20 mM piperazine-HCl, pH 5.0. The anion exchange chromatography (Q FF column, 1.6×10 cm) was performed by applying the reconstituted protein and the flow-through fraction was applied to the lysophosphatidylethanolamine affinity column. The purified L-FABP was saturated with 10-times molar oleic acid and dialyzed against saline. Purity was assessed by SDS-PAGE, i.e. the protein was appeared as a single band to silver staining. N-terminal amino acid analysis yielded only one N-terminus.

Measurement of ROS
The oxidative fluorescence dihydroethidium (DHE) was used to evaluate intracellular O$_2^-$ levels as described previously\textsuperscript{6}. Briefly, mProx with or without pretreatment of rhL-FABP (1mmol/L, 4h) and mProx-L were plated in 6-well plates. At the appropriate time after stimulation, dihydroethidium (4 mol/L) was added to the medium, and the incubation was continued for 30 min. Then, the cells were washed with PBS and the images were obtained with a laser scanning confocal microscope system (FLUOVIEW FV1000; Olympus, Tokyo, Japan). The averages of fluorescence intensity values from 10 to 15 cells of 3 to 5 different examinations were calculated using the KS400 version 3.0 image analysis system (Carl Zeiss Vision GmbH, Germany).

AGT and MCP-1 ELISA
Mouse AGT (code no. 27413, IBL, Gumma, Japan) and MCP-1 (R&D systems, Minnesota, USA) ELISA was performed to quantify mouse AGT and MCP-1 protein in the culture medium. mProx and mProx-L were cultured in 6 mm dish with 3.0 ml/dish of medium. After the treatment of cells with AngII (10\textsuperscript{-7} mol/L) or AngII with rhL-FABP pretreatment (1mmol/L, 12h), the medium was collected at 12 h.

Statistical analysis
All numerical data are expressed as the mean ± SD or mean ± SEM. Differences among experimental groups were determined using one-way analysis of variance with Fisher’s post hoc analysis. A p-value < 0.05 was considered as statistically significant. The analyses were performed using StatView 5.0 software (Abacus Concepts, Inc., Stanford, CA, USA).
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