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ORIGINAL PAPER

Urinary FABP1 is a biomarker for impaired proximal tubular protein reabsorption and is synergistically enhanced by concurrent liver injury

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Abstract

Urinary fatty acid binding protein 1 (FABP1, also known as liver-type FABP) has been implicated as a biomarker of acute kidney injury (AKI) in humans. However, the precise biological mechanisms underlying its elevation remain elusive. Here, we show that urinary FABP1 primarily reflects impaired protein reabsorption in proximal tubule epithelial cells (PTECs). Bilateral nephrectomy resulted in a marked increase in serum FABP1 levels, suggesting that the kidney is an essential organ for removing serum FABP1. Injected recombinant FABP1 was filtered through the glomeruli and robustly reabsorbed via the apical membrane of PTECs. Urinary FABP1 was significantly elevated in mice devoid of megalin, a giant endocytic receptor for protein reabsorption. Elevation of urinary FABP1 was also observed in patients with Dent disease, a rare genetic disease characterized by defective megalin function in PTECs. Urinary FABP1 levels were exponentially increased following acetaminophen overdose, with both nephrotoxicity and hepatotoxicity observed. FABP1-deficient mice with liver-specific overexpression of FABP1 showed a massive increase in urinary FABP1 levels upon acetaminophen injection, indicating that urinary FABP1 is liver-derived. Lastly, we employed transgenic mice expressing diphtheria toxin receptor (DT-R) either in a hepatocyte- or in a PTEC-specific manner, or both. Upon administration of diphtheria toxin (DT), massive excretion of urinary FABP1 was induced in mice with both kidney and liver injury, while mice with either injury type showed marginal excretion. Collectively, our data demonstrated that intact PTECs have a considerable capacity to reabsorb liver-derived FABP1 through a megalin-mediated mechanism. Thus, urinary FABP1, which is synergistically enhanced by concurrent liver injury, is a biomarker for impaired protein reabsorption in AKI. These findings address the use of urinary FABP1 as a biomarker of histologically injured PTECs that secrete FABP1 into primary urine, and suggest the use of this biomarker to simultaneously monitor impaired tubular reabsorption and liver function.

Keywords: urinary FABP1; proximal tubule epithelial cells; protein reabsorption; acute kidney injury; liver injury; megalin; Dent disease

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No conflicts of interest were declared.

Introduction

Acute kidney injury (AKI) is a common and life-threatening condition that is frequently accompanied by multiple organ dysfunction, including liver injury [1–4]. Early detection of AKI is crucial for timely intervention and prevention of complications. Measurements of kidney function by serum creatinine, urine output, or urinalysis are inadequate in their ability to detect AKI, which has a tubulointerstitial pathology. Urinary proteins have been described as biomarkers for renal tubule dysfunction or injury [1–4]. The major biomarkers include kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and fatty acid binding protein 1 (FABP1); together, these markers enable a more accurate and earlier diagnosis of AKI in humans, and clinical validation of these biomarkers has been advanced [1–4].

FABP1 (also known as liver type-FABP, L-FABP) is a 14 kDa cytosolic protein that is predominantly expressed in the liver and, to a lesser extent, in the kidney, intestine, stomach, and lung [5,6]. Urinary FABP1 levels are elevated in several acute kidney diseases such as ischemia reperfusion injury/hypoperfusion, acute decompensated heart failure [7–9], cardiac surgery [10], kidney transplantation [3,11], sepsis [12], and contrast- [13,14] and nephrotoxin-induced AKI [3,15]. Several studies have reported that urinary FABP1 is useful for the early detection and prediction of prognosis [3,4,11,16–19]. Because FABP1 expression is low in the kidneys of mice, transgenic mice expressing human FABP1 in proximal tubule epithelial cells (PTECs) have been used to examine urinary FABP1 after kidney injury [20–25]. The results of those experiments suggest that FABP1 is excreted from proximal tubules (PTs) in response to renal insults, and thus urinary FABP1 has been proposed as a biomarker for PT injury. However, the precise pathophysiological pathways leading to increased urinary excretion of FABP1 remain poorly defined.

Some studies have shown that urinary FABP1 levels are concomitantly increased with markers of hepatic injury (aspartate aminotransferase, AST, and alanine aminotransferase, ALT) and hypoperfusion (lactate) but not serum creatinine in patients admitted to the intensive care unit [26]. Other studies have shown that urinary FABP1 levels do not increase in patients with liver dysfunction alone [27] or nephrotic syndrome with minimal glomerular abnormalities [28,29]. It has also been shown that an increase in urinary FABP1 levels is not associated with elevated serum FABP1 levels in patients with septic shock [30] or in those who have undergone cardiac surgery [31]. These conflicting results prompted us to determine the precise pathophysiological mechanisms underlying the elevation of urinary FABP1 levels.

In this study, we tested the hypothesis that circulating FABP1 derived from the liver is almost completely removed by glomerular filtration and subsequently reabsorbed by PTECs. We also tested whether an increase in the release of FABP1 from hepatocytes is associated with an elevation in urinary FABP1 by employing two distinct models: FABP1-deficient mice with liver-specific overexpression of FABP1, and mice expressing diphtheria toxin receptor (DT-R) in a PTEC- or hepatocyte-specific manner. We show here that urinary FABP1 is derived from circulating FABP1 released from hepatocytes and that DT-induced kidney-specific or liver-specific injury alone is not necessarily sufficient for an increase in urinary FABP1 levels. We further show that urinary FABP1 is reabsorbed by PTECs through a megalin-mediated mechanism. Our data strongly suggest that urinary FABP1 is a reliable biomarker for dysfunctional protein reabsorption by PTECs, which is synergistically enhanced by liver injury.

Materials and methods

Human sample analysis

The human study was approved by the Institutional Review Board of Niigata University (2017-0108) in accordance with the principles embodied in the Declaration of Helsinki, and all participants provided written informed consent.

Animal models

All animal studies were approved by the Institutional Animal Care and Use Committee (Gunma University Graduate School of Medicine, 18-028 and 20-001) and conformed to the National Institute of Health guidelines (Guide for the Care and Use of Laboratory Animals). Global *Fabp1*$^{-/-}$ mice were generated using Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 (CRISPR/Cas9) genome-editing technology and pronuclear stage embryos as described previously [32–34] (see Supplementary materials and methods). Cre recombinase (Cre)-inducible diphtheria toxin receptor (iDTR) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *Ndrg1*$^{CreERT2/+}$ mice were crossed with iDTR mice to generate *Ndrg1*$^{CreERT2/+}$*:iDTR* mice [35,36]. The PCT injury model was produced as described in Supplementary materials and methods. PTEC-specific megalin KO mice (*Ndrg1*$^{CreERT2/+}$*:megalin*$^{fl/fl}$) with a C57BL/6J background were generated as described elsewhere [37]. The mice were housed in a temperature-controlled room (20–26 °C) with a 12-h light/12-h dark cycle and given unrestricted access to water and standard chow (CE-2; Clea Japan Inc, Tokyo, Japan). Bilateral or unilateral nephrectomy (BLN or ULN, respectively) and sham operations were performed as described in Supplementary materials and methods [38]. Acetaminophen (APAP) was intraperitoneally injected at a dose of 200 mg/kg after a 12-h fast to induce kidney and liver injury. Blood was collected from the retro-orbital venous plexus 24 h after injection [39,40].

AAV vectors

Adeno-associated virus (AAV) vector was constructed for gene delivery in mice. The plasmid containing the

albumin (*Alb*) promoter (2.33 kb) was purchased from Addgene (Watertown, MA, USA). FABP1 cDNA was isolated by PCR. The plasmid encoding hHBegf was provided by Dr H Akazawa [41]. Three plasmids were constructed to express GFP, FABP1, and hHBegf under the control of the albumin promoter (pAAV-Alb-GFP, pAAV-Alb-FABP1, and pAAV-Alb-hHBegf). Recombinant single-strand AAV serotype 9 (AAV9) particles were produced using the ultracentrifugation method described previously [42]. In brief, three plasmids, pHelper (Stratagene, La Jolla, CA, USA), pAAV2/9 (provided by Dr J Wilson, University of Pennsylvania), and one pAAV-Alb plasmid, were co-transfected into human embryonic kidney (HEK) 293T cells (HCL4517; Thermo Fisher Scientific, Waltham, MA, USA) using polyethylenimine (24765-1; Polysciences, Inc, Warrington, PA, USA). Viral particles were harvested from the culture medium 6 days after transfection and concentrated by precipitation with 8% polyethylene glycol 8000 (Sigma-Aldrich, St Louis, MO, USA) and 500 mM sodium chloride. The precipitated AAV particles were resuspended in Dulbecco's phosphate-buffered saline (D-PBS) and purified with iodixanol (OptiPrep; Axis-Shield Diagnostics, Dundee, UK) continuous gradient centrifugation. The viral solution was further concentrated in D-PBS using Vivaspin 20 (100,000 MWCO PES; Sartorius, Göttingen, Germany). The genomic titers of the viral vector were determined by real-time quantitative PCR using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) with the primers 5'-CTGTTG GGCACTGACAATTC-3' and 5'-GAAGGGACGTAG-CAGAAGGA-3', which targeted the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) sequence. The expression plasmid was used as the standard. The PCR cycling parameters were as follows: initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The phenotype was analyzed 4 weeks after injection of AAV vectors into the retro-orbital venous plexus.

Immunofluorescence analysis

Recombinant human FABP1 (Cayman Chemical, Ann Arbor, MI, USA) was labeled with Alexa Fluor 647 (AF647) reactive dye (Thermo Fisher Scientific) according to the manufacturer's protocol. Ten minutes after intravenous injection of AF647-FABP1 (15 µg per mouse in 200 µl of saline) or AF647 alone, the kidneys were isolated, cut into two equal halves, fixed in 10% buffered formalin or Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 24 h, and then embedded in paraffin. Immunofluorescence analysis was performed with fluorescein-labeled lotus tetragonolobus lectin (LTL; Vector Laboratories, Burlingame, CA, USA), anti-FABP1 antibody (Cell Signaling Technology, Inc, Danvers, MA, USA), and AF555-labeled anti-rabbit IgG (Thermo Fisher Scientific). Images for immunofluorescence analysis were captured using a BZ-9000 microscope (Keyence Corporation, Osaka, Japan).

Measurement of FABP1/4 and biochemical parameters in serum and urine

FABP1 and FABP4 levels in serum and urine were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (R&D Systems, Inc, Minneapolis, MN, USA) and Bio-Vendor (Brno, Czech Republic), respectively. Serum and urine biochemical parameters were measured by Oriental Yeast Co, Ltd, Tokyo, Japan.

RNA isolation and RT-qPCR

Total RNA isolation, reverse transcription, and quantitative real-time PCR were performed as previously described [43,44]. The expression level of the target gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA. Ready-to-use gene-specific primers for cDNA (perfect real-time primers) were purchased from Takara Bio Inc, Shiga, Japan.

Western blot analysis

Western blot analysis was performed with antibodies against FABP1 (Cell Signaling Technology, Inc) and human HBegf (Abcam, Cambridge, UK) as described previously [43,44].

Statistical analysis

Statistical analyses were performed using IBM SPSS (version 26; IBM, New York, NY, USA). The data are presented as mean ± standard deviation or median ± interquartile range, depending on the normality of the distribution. Student's *t*-test or a Mann–Whitney test was performed for the two groups as appropriate. One-way analysis of variance (ANOVA) with Tukey's *post hoc* multiple comparison tests was performed for three groups. Spearman's simple correlation analyses were performed to determine the association between urinary FABP1 levels and biochemical parameters in serum and urine. Two-way ANOVA was used to analyze the two main effects and their interactions. Statistical significance was set at $p < 0.05$. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; main effect for one. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; main effect for the other. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$; interaction between the two main effects.)

Results

Kidneys are essential for the clearance of serum FABP1

To determine whether renal clearance affects serum FABP1 levels, bilateral or unilateral nephrectomy was performed. The serum levels of FABP1 and creatinine were markedly elevated 12 h after BLN but not after ULN (Figure 1A). These findings suggest that the kidney is an essential organ for the clearance of circulating FABP1.

Serum FABP1 is reabsorbed via the apical membrane of PTECs

We recently reported that recombinant FABP4 (also known as adipocyte-FABP) is filtered through the glomeruli and reabsorbed by PTs [38]. We next studied whether intravenously injected recombinant human FABP1 (rFABP1) is similarly cleared by the kidney like rFABP4. As expected, a marked reabsorption of rFABP1 was observed in PTs 10 min after

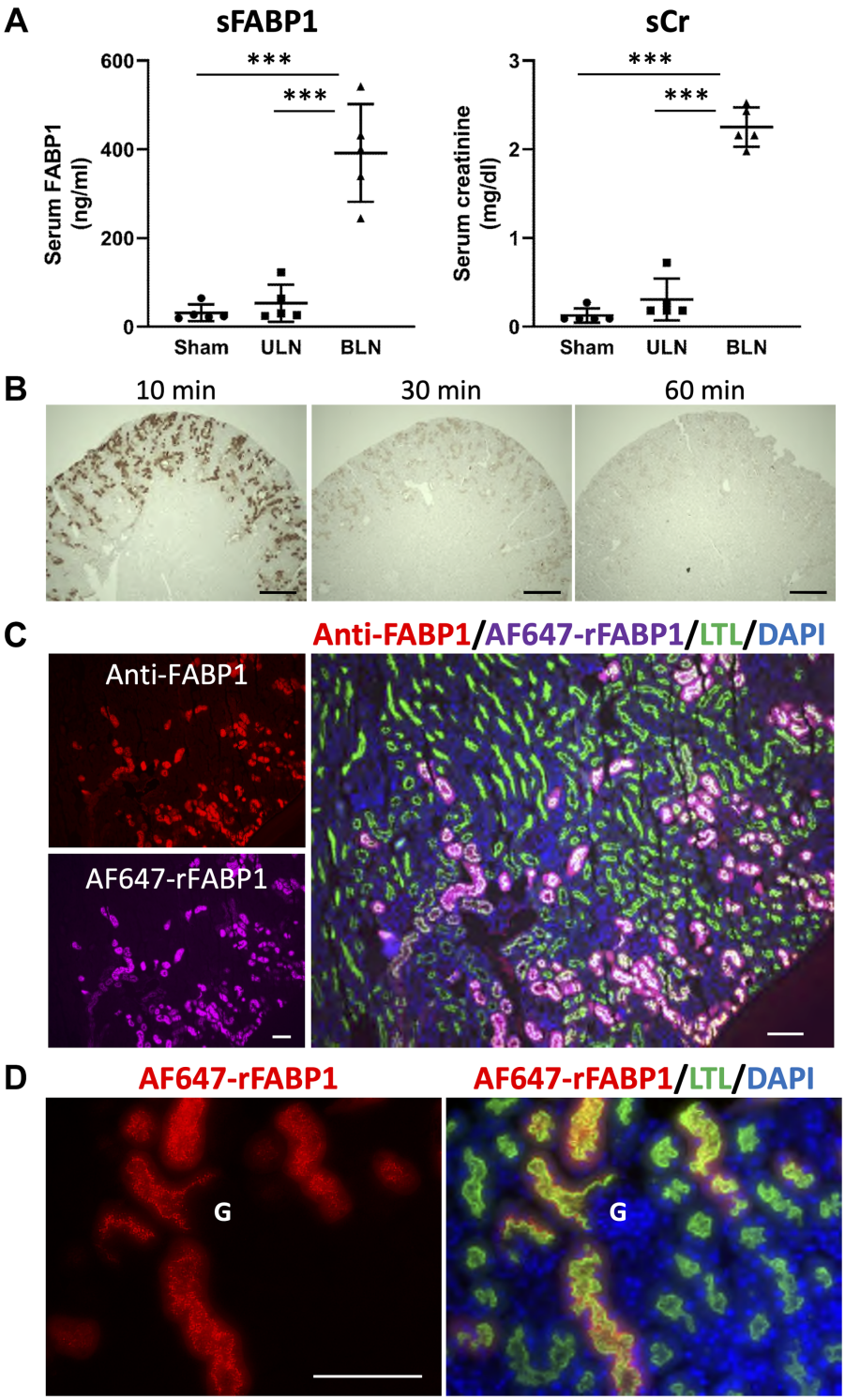

Figure 1. Circulating FABP1 is reabsorbed by PTECs. (A) Serum FABP1 levels were elevated by BLN but not by ULN. Blood was collected from the retro-orbital plexus 12 h after surgery to measure the serum levels of creatinine and FABP1 ($n = 5$). ***$p < 0.001$. BLN, bilateral nephrectomy; ULN, unilateral nephrectomy. (B) The kidney was isolated 10, 30, and 60 min after intravenous injection of recombinant human FABP1 (rFABP1) for subsequent immunohistochemistry. Scale bar = 500 μm. (C, D) The kidney was isolated 10 min after intravenous injection of Alexa Fluor 647 (AF647)-rFABP1 for immunofluorescence. Scale bar = 100 μm. (C) Injected AF647-rFABP1 was detected by anti-FABP1 antibody (red) and AF647 (purple). (D) Higher magnification. AF647-rFABP1 (red); lotus tetragonolobus lectin (LTL), a marker for brush border of PTECs (green); DAPI, 4',6-diamidino-2-phenylindole, a marker for nuclei (blue).

injection and declined thereafter (Figure 1B). The distribution of AF647-labeled recombinant FABP1 (AF647-rFABP1) overlapped with that detected by an antibody against FABP1 (Figure 1C). AF647-rFABP1 was exclusively reabsorbed via the apical membrane of PTECs, which were detected by LTL, a marker for PTECs (Figure 1C,D). AF647-rFABP1-positive particles were also detected just below the apical membrane but not throughout the cytoplasm (Figure 1D), suggesting that AF647-rFABP1 is taken up into endocytic vesicles [45]. These findings suggest that serum FABP1 is filtered through the glomeruli and endocytosed by PTECs.

Megalin-mediated endocytosis is responsible for reabsorption of FABP1 by PTECs

Next, we attempted to identify the molecules involved in the reabsorption of FABP1 in PTECs. Oyama *et al* reported that FABP1 is taken up by the kidney through interaction with the apical membrane protein megalin [46]. It was also reported that a large amount of FABP4 and albumin, other megalin ligands, was excreted into the urine in megalin KO mice [38,47]. Accordingly, we postulated that megalin mediates FABP1 reabsorption in PTECs. As expected, FABP1 levels in urine were markedly elevated in megalin KO mice compared with WT mice (Figure 2). Furthermore, urinary FABP1 levels were remarkably increased in patients with Dent disease, whose function for proximal tubular megalin-mediated endocytosis is impaired, as evidenced by elevated levels of urinary α1- and β2-microgloblin (39 and 14 kDa, respectively; two established megalin ligands) (supplementary material, Table S1) [45,47,48]. These findings suggest that megalin expressed in the apical membrane of PTECs is responsible for the reabsorption of FABP1 following glomerular filtration.

An excessive dose of acetaminophen induces a massive increase in urinary FABP1 predominantly by kidney injury

Overdose of acetaminophen (APAP; 4'-hydroxyacetanilide, 4-acetamidophenol, *N*-acetyl-4-aminophenol), an analgesic drug, induces both hepatotoxicity and nephrotoxicity (acute tubular necrosis) [39,40]. Intraperitoneal injection of APAP induces severe liver injury with a considerable increase in AST and ALT levels (Figure 3), which was accompanied by a variable increase in serum FABP1 levels (median: 9.7 versus 184.6). Serum creatinine levels were modestly elevated, while those in urine were decreased (Figure 3), which strongly suggests that glomerular filtration was also disturbed. Surprisingly, urinary FABP1 levels were exponentially increased (median: 13.3 ng/day versus 238 μg/day). Compared with urinary FABP1, the elevation in FABP4 and albumin levels in urine was modest (Figure 3). Interestingly, urinary FABP1 levels were positively associated with FABP4 and albumin levels in urine but not with serum FABP1 levels (supplementary material, Table S2). These findings suggest that there are both common and distinct mechanisms underlying the excretion of FABP1/4 and albumin into urine, and that one of the more common mechanisms could be impaired reabsorption. The expression of *Fabp1* mRNA in the kidney was increased 3.1-fold following APAP treatment. Because the expression of FABP1 in the liver was 6000-fold higher than that in the kidney at baseline (Figure 4A), the contribution of kidney-derived FABP1 was negligible in urinary FABP1. Furthermore, it is noteworthy that a large amount of FABP1 was detected by both western blot (Figure 4B) and immunofluorescence (Figure 4D) in APAP-treated mice. Interestingly, a robust increase in FABP1 protein in the kidney was accompanied by a considerable increase in serum FABP1 levels (Figure 4C), suggesting that reabsorption of FABP1 in the kidney results from an increase in serum FABP1 levels.

Urinary FABP1 is derived from circulating FABP1 released from hepatocytes

To prove that urinary FABP1 caused by APAP-induced injury is exclusively derived from hepatocytes, we next performed the same APAP administration experiments using

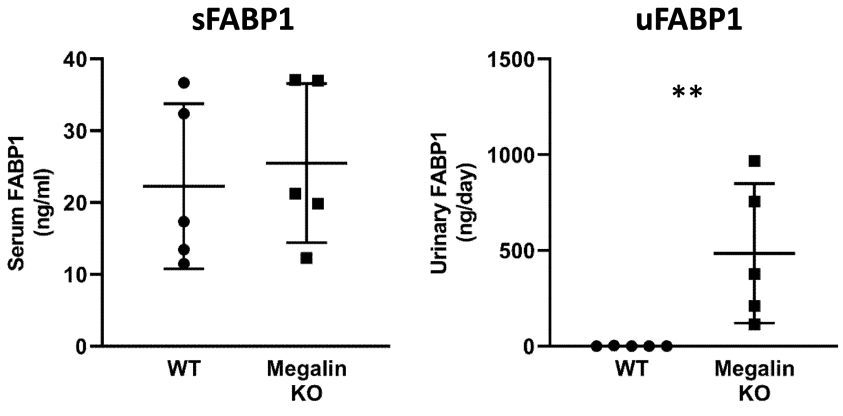

Figure 2. Megalin-mediated reabsorption of FABP1 in PTECs. FABP1 is excreted into urine in PTEC-specific megalin KO mice (*Ndrg1*$^{CreERT2/+}$*: megalin flox/flox*). FABP1 levels in serum (left panel) and urine (right panel) in megalin KO mice. $n = 5$. $^{**}p < 0.01$.

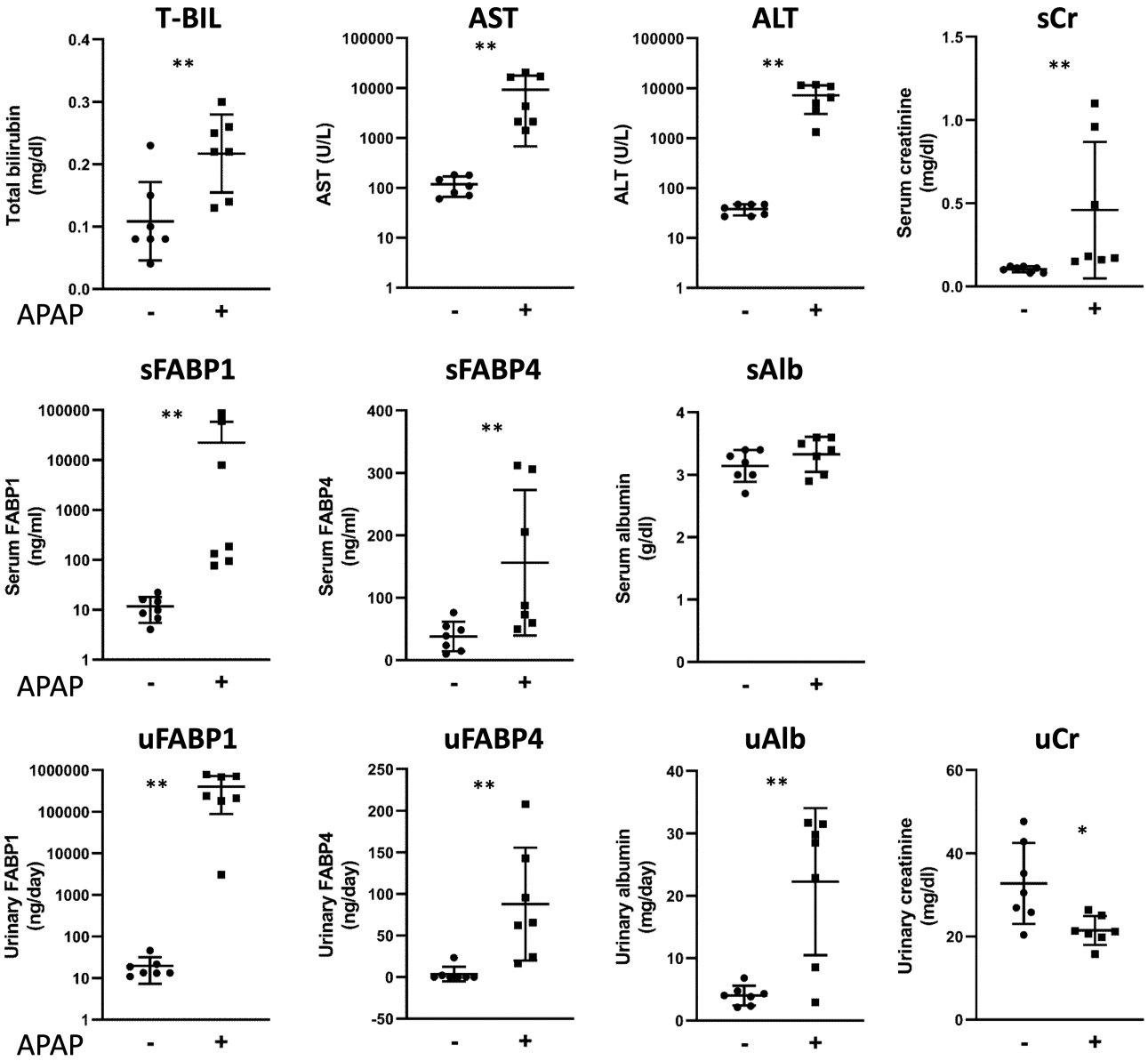

Figure 3. Considerable excretion of FABP1 into urine in APAP-induced hepatotoxicity and nephrotoxicity model. Acetaminophen (APAP) was intraperitoneally injected at 200 mg/kg after a 12-h fast. Blood sampling was performed from the retro-orbital plexus 24 h after the injection, while urine was collected for 24 h for subsequent biochemical analysis ($n = 7$). Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; T-BIL, total bilirubin.

FABP1-deficient mice with liver-specific overexpression of FABP1 using AAV9 vectors. Exclusive expression of FABP1 driven by the albumin promoter was confirmed by western blot in the livers of FABP1 KO mice (Figure 5A, lane 3). As expected, both serum and urine levels of FABP1 were markedly elevated by APAP overdosing when FABP1 was overexpressed exclusively in the liver (Figure 5B), indicating that urinary FABP1 is derived from the liver. Although intravenously injected rFABP1 was robustly reabsorbed via PTECs in FABP1-deficient kidneys (Figure 5C, left panel), the strong accumulation was markedly diminished by APAP treatment (Figure 5C, right panel). These findings strongly support our hypothesis that urinary FABP1 originates from liver-derived FABP1 in serum and is increased by impairment of its reabsorption in PTECs.

Concurrent disruption of hepatocytes and PTECs increases urinary FABP1

To determine the mechanisms of urinary excretion of FABP1 more precisely, we next employed mice expressing diphtheria toxin receptor (DT-R; or human heparin-binding epidermal growth factor, hHBegf) in either a PTEC- or a hepatocyte-specific manner, which was driven by N-myc downregulated gene 1 (*Ndrg1*)- or *Alb* promoters, respectively (Supplementary materials and methods and supplementary material, Figure S1A). In these mice, hepatocytes and/or PTECs were subjected to damage upon intraperitoneal administration of DT (supplementary material, Figure S1B). Selective damage in either PTECs or hepatocytes led to only a marginal increase in urinary FABP1

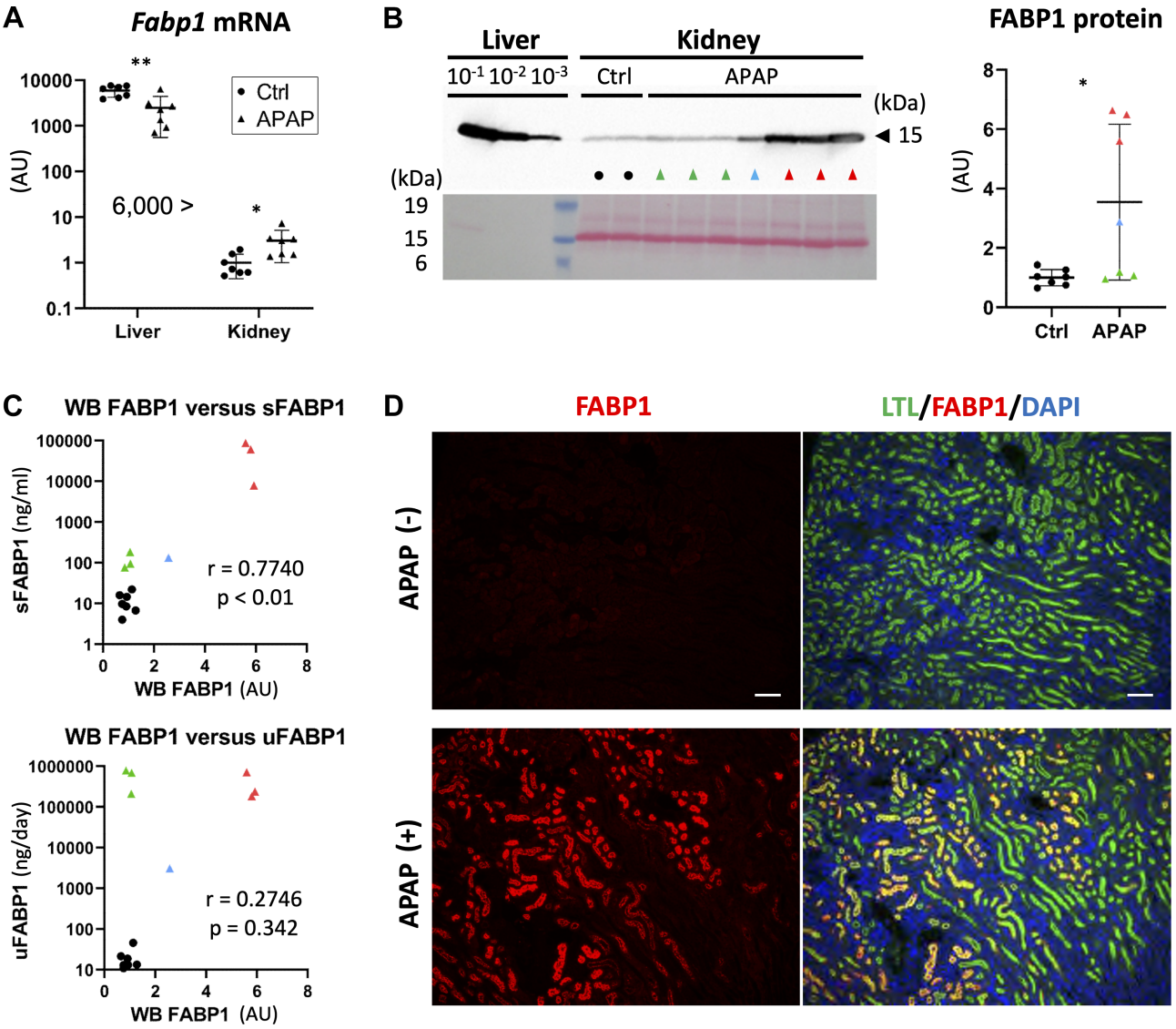

Figure 4. Reabsorption of FABP1 in PTECs is associated with serum FABP1 levels. The samples in Figure 3 were further analyzed. (A) Expression of *Fabp1* mRNA in the liver and kidney was determined by RT-qPCR ($n = 7$). (B) Accumulation of FABP1 protein in the kidney was detected by western blotting (WB) ($n = 7$). (C) The amounts of FABP1 protein in the kidney relative to those in serum (upper panel) and in urine (lower panel). (D) Immunofluorescence of kidney 24 h after APAP injection. Scale bar = 100 μm. LTL (green); rFABP1 (red); DAPI (blue). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

levels (Figure 6A). However, when both PTECs and hepatocytes were simultaneously damaged, the amount of urinary FABP1 was synergistically elevated (Figure 6A). Expression of *Fabp1* mRNA in the kidney was markedly reduced in PTEC-specific injury mice (Figure 6B), strongly suggesting that urinary FABP1 is not derived from endogenously produced FABP1 in PTECs. FABP1 was highly reabsorbed via PTECs in a hepatocyte-specific injury model, where an elevation in serum FABP1 was observed (Figure 6A,C, upper panel). When both PTECs and hepatocytes were simultaneously damaged, reabsorption of FABP1 was diminished in early segments of PTECs but was maintained in late segments (Figure 6C, lower panel). These data suggest that there is a large capacity for protein reabsorption in the PTECs at baseline, and that urinary FABP1 levels are increased when the FABP1 flux into the nephron exceeds the residual capacity of protein reabsorption by kidney injury (Figure 6E; see Discussion).

Discussion

Although urinary FABP1 has been identified as a sensitive biomarker for tubulointerstitial damage, the pathophysiological pathways contributing to its elevation remain elusive. Theoretically, there are at least four factors that influence urinary FABP1 levels: liver-derived FABP1 levels in serum, selectivity of glomerular filtration of FABP1, protein reabsorption by PTECs, and secretion from PTECs (Figure 6D). Among them, few studies have examined the effects of glomerular

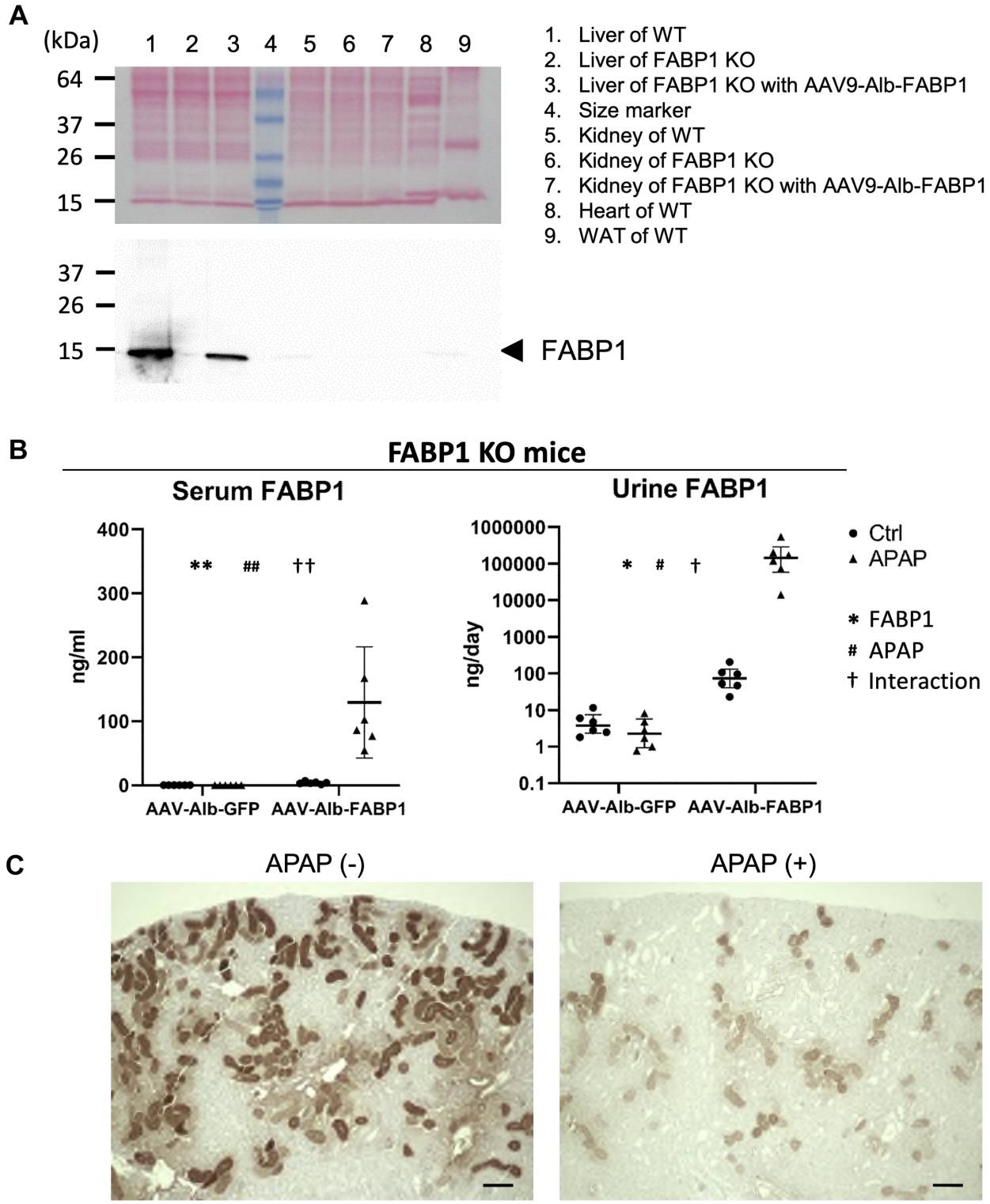

Figure 5. Urinary FABP1 originates in the liver. (A) Absence of FABP1 expression in the liver in FABP1-deficient mice and its overexpression by an adeno-associated virus 9 (AAV9) vector, AAV9-Alb-FABP1, were confirmed by western blotting. (B) Four weeks after retro-orbital injection of AAV9-Alb-FABP1 or AAV9-Alb-GFP at a dose of 1.0E+9 viral genome (vg) per mouse, an APAP-induced tissue injury model was produced as described in Figure 3. FABP1 in serum and urine was measured ($n = 6$). Note that the FABP1 levels in serum and urine were markedly elevated by APAP treatment when FABP1 was overexpressed in the liver. $^{*,\#,\dagger}p < 0.05$, $^{**,\#\#,\dagger\dagger}p < 0.01$. (C) Ten minutes after intravenous injection of rFABP1 in FABP1-deficient mice in the absence or presence of APAP treatment. FABP1 reabsorption was detected by immunohistochemistry. Scale bar = 100 μm. Note that rFABP1 reabsorption was reduced by APAP treatment.

filtration and reduced protein reabsorption on urinary FABP1 levels. In this study, we demonstrated that a reduction in protein reabsorption in PTECs is the most important determinant of urinary FABP1, which is predominantly derived from the liver. Serum FABP1 levels affect urinary FABP1 excretion when the

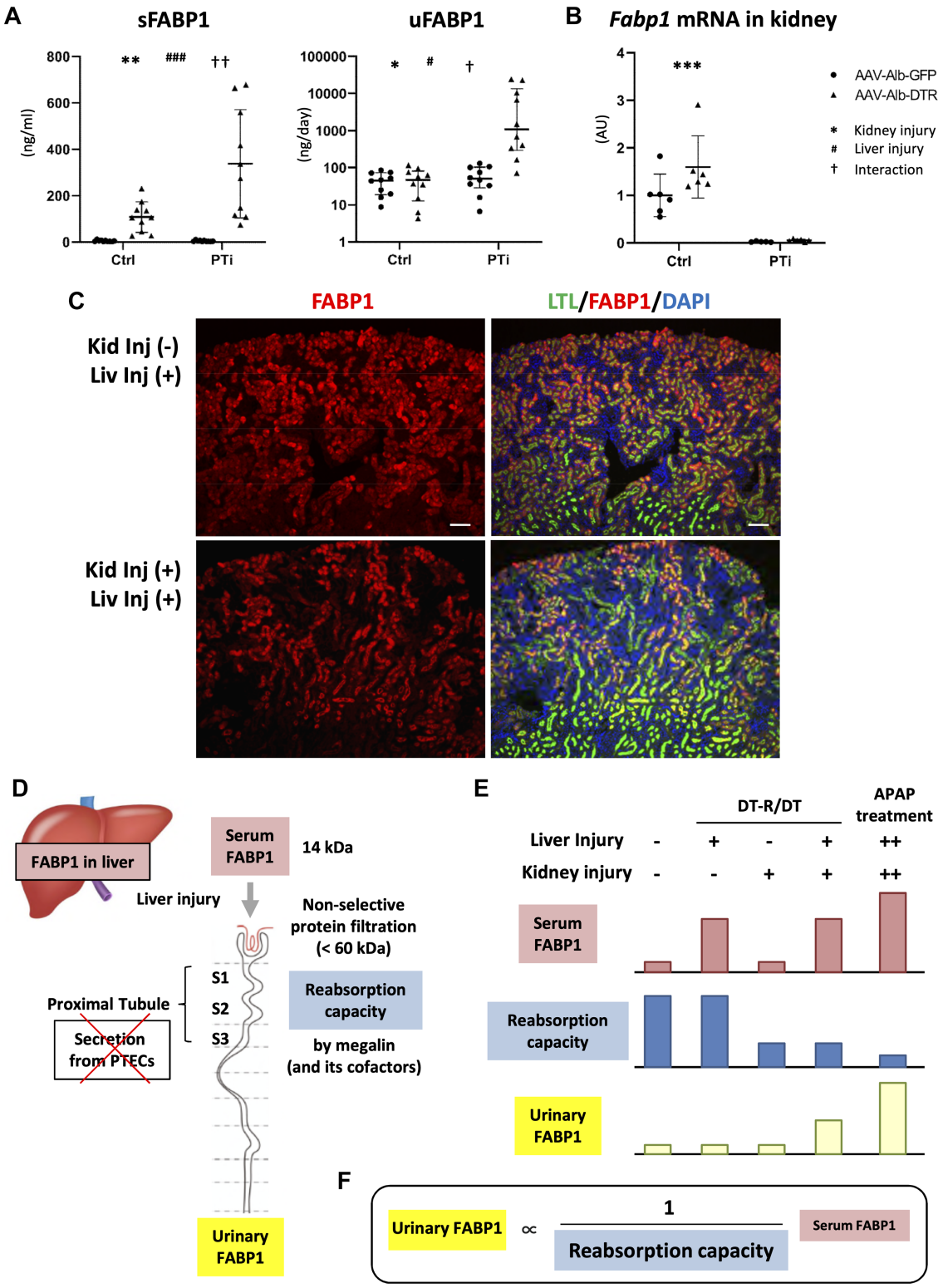

Figure 6. Elevation of urinary FABP1 levels by concurrent kidney and liver injury. (A) Kidney-specific injury was induced in *Ndrg1*$^{CreERT2/+}$*:iDTR* mice by intraperitoneal (i.p.) administration of diphtheria toxin (DT). iDTR represents Cre recombinase (Cre)-inducible diphtheria toxin receptor. Liver-specific injury was induced by overexpression of hHBegf driven by the albumin promoter using AAV9 with subsequent i.p. administration of DT. Concurrent kidney and liver injury was induced by a combination of the two models (see Materials and methods and Supplementary materials and methods). Blood was collected from the retro-orbital plexus 48 h after DT injection, while urine was collected for 24 h (24–48 h after i.p. administration of DT). The FABP1 levels in serum and urine were measured by ELISA ($n = 10$). $^{*,\#,\dagger}p < 0.05$, $^{**,\dagger\dagger}p < 0.01$, $^{\#\#\#}p < 0.001$. (B) Expression of *Fabp1* mRNA in the kidney was strongly suppressed by proximal tubule injury (PTi) ($n = 6$). $^{***}p < 0.001$. (C) Distribution of endogenous FABP1 in the kidney in the liver injury model with or without kidney injury. Scale bar = 100 μm. LTL (green); FABP1 (red); DAPI (blue). (D–F) Schematic representation of the mechanism underlying elevation of urinary FABP1. (D) Reabsorption capacity by PTECs is a major determinant of urinary FABP1, followed by serum FABP1 levels (see Discussion). (E) Relationship between urinary FABP1 and serum FABP1/reabsorption capacity in each experimental group in this study. (F) Urinary FABP1 levels are associated with the inverse of protein reabsorption capacity and serum FABP1 levels.

reabsorption capacity is diminished. In addition, an increase in urinary FABP1 in megalin KO mice and in patients with Dent disease, a rare genetic disease characterized by dysfunction of proximal tubular endocytosis, provided compelling evidence that megalin-mediated reabsorption in PTECs is a critical determinant of urinary FABP1.

Contribution of serum FABP1 levels to urinary FABP1

A liver-specific injury model in mice carrying the DT-R/DT system demonstrated that an elevation in serum FABP1 levels resulted in a marginal elevation in urinary FABP1 (Figure 6A). Consistent with this, a previous study reported that urinary FABP1 levels were not increased in patients with liver diseases alone [27]. These data demonstrated that serum FABP1 levels are not directly linked to urinary FABP1 levels under normal renal function, due to the large reabsorption capacity (Figure 6E). When the reabsorption capacity is reduced by kidney injury or defective function of megalin in PTECs, serum FABP levels finally influence urinary FABP1 (Figure 6A,E).

Contribution of impaired glomerular filtration to urinary FABP1

Low-molecular-weight proteins (<60 kDa) were found to be filtered through glomeruli [49] (Figure 6D), implying that the concentration of FABP1 in primary urine is proportional to that in serum FABP1 levels. Indeed, FABP1 reabsorption was found in PTECs in normal kidneys when serum FABP1 levels were increased (e.g. rFABP1 i.v. and liver-specific injury by DT-R/DT system) (Figures 1B–D, 5C, and 6C). Consistently, in patients with minimal change nephrotic syndrome or minor glomerular abnormalities, urinary FABP1 levels do not increase despite a marked increase in urinary albumin [28,29]. These observations reinforce our argument that a large amount of filtered FABP1 is efficiently reabsorbed in PTECs unless proximal tubular endocytosis is impaired (Figure 6E).

Contribution of the impaired protein reabsorption in PTECs to urinary FABP1

Our finding that urinary FABP1 is increased in megalin KO mice and in patients with Dent disease (Figure 2 and supplementary material, Table S1) revealed that megalin-mediated protein reabsorption is a critical determinant of urinary FABP1. In addition, in mice treated with APAP, urinary FABP1 was disproportionately increased compared with serum FABP1 levels (median: 18 000-fold versus 19-fold elevation). These results suggest a closer association between urinary FABP1 levels and a reduction in protein reabsorption capacity, rather than serum FABP1 levels (Figures 3 and 4, and supplementary material, Table S2). A large capacity for protein reabsorption was demonstrated by a marginal increase in urinary FABP1 and overt accumulation of FABP1 in PTECs by the liver-specific DT-R/DT model (Figure 6A,C). The increase in urinary FABP1 was modest in mice with both kidney and liver injury (Figure 6A) compared with that in the APAP-induced injury model. This could be explained by the residual reabsorption capacity in the late segments of PTECs because the promoter activity of *Ndrg1* is weak in late segments [35]. Thus, it is likely that urinary FABP1 levels are primarily determined by the protein reabsorption function in PTECs and, to a lesser extent, by serum FABP1 levels (Figure 6D,E).

Influence of excretion of FABP1 from PTECs on urinary FABP1

It has been postulated that FABP1 is excreted from PTECs to urine when PTECs are subjected to nephrotoxic agents or ischemia–reperfusion injury [20–25]. This hypothesis is based on the assumption that FABP1 binds to reactive oxygen species (ROS)-induced lipid peroxidation products and transfers them to the urinary space in hypoxic PTECs. Because expression of FABP1 is lower in PTECs in mice than in humans, transgenic mice expressing human FABP1 driven by the promoter region (13 kb) have been developed as a model mimicking the human kidney [25]. Although these studies showed an increase in urinary FABP1 levels upon renal insults, the question of whether endogenous FABP1 in PTECs is excreted into urine remains unanswered.

Here, we demonstrated that *Fabp1* mRNA levels in the mouse kidney were 6000-fold lower than those in the liver at baseline (Figure 4A) and were increased by merely 3.1-fold in the kidney in the APAP-treated mouse model. Nevertheless, both FABP1 protein levels in PTECs and urinary FABP1 were robustly increased in this model. In the combined kidney and liver injury model using the DT-R/DT system, urinary FABP1 levels were significantly increased, although *Fabp1* mRNA levels were markedly suppressed (Figure 6A,B). These data clearly contradict the reported hypothesis that FABP1 expression is induced in PTECs and that FABP1 is excreted into primary urine in response to oxidative stress generated by nephrotoxic insults. Instead, our data provide evidence that urinary FABP1 and reabsorbed FABP1 in PTECs are derived from serum FABP1 released from hepatocytes.

Clinical perspectives of our findings

Several urinary proteins have been identified as early biomarkers for detecting renal tubular damage in patients with AKI. KIM-1, a putative adhesion molecule that contains extracellular immunoglobulin-like domains, is expressed at a low level in normal kidneys but is markedly induced in regenerating PTECs in ischemic and nephrotoxic AKI animal models [1–4]. Likewise, NGAL, an acute phase reactant released from immune cells, is dramatically induced mainly in the distal nephron after ischemic and toxic kidney injury in human and animal models [1–4]. Therefore, the pathophysiological pathway that leads to an increase in urine

protein concentration upon AKI should be distinct from that in FABP1. To the best of our knowledge, our study is the first to demonstrate that urinary FABP1 reflects impaired protein reabsorption during tubular injury, which is enhanced by concurrent liver injury. Further studies are warranted to assess the diagnostic performance of urinary FABP1 in patients with AKI and chronic kidney disease.

In summary, we propose the following model (Figure 6D–F): In principle, urinary FABP1 is derived from serum FABP1 released from hepatocytes. When solely liver injury is introduced, most of the FABP1 influx into the nephron can be reabsorbed. When liver injury occurs concurrently with kidney injury, FABP1 levels in primary urine exceed the residual reabsorption capacity, leading to an elevation in urinary FABP1 levels. Severe kidney injury with minor or no liver injury can also cause an increase in urinary FABP1 levels. Thus, urinary FABP1 levels are associated with the inverse of protein reabsorption capacity and serum FABP1 levels (Figure 6F). Because kidney injury is frequently accompanied by liver injury in many clinical settings, it is plausible that mutual effects enhance urinary FABP1 levels. We conclude that urinary FABP1 is a biomarker for impaired protein reabsorption in AKI, which is synergistically enhanced by liver injury. Our model of urinary FABP1 elevation could contribute to our understanding of the pathophysiology of AKI with multiple organ dysfunction, including liver injury, and to the development of novel strategies to detect and treat these patients.

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Author contributions statement

TI designed the experiments. RK, MM, SS, SS, HS, HH, SG, MH, HK, MO, NK AS, MY, HH and TI conducted the majority of the investigations. RK generated the FABP1-deficient mice and AK and HH produced the AAV vectors. RK and TI analyzed the data. RK, MK and TI drafted the manuscript. RK, TS, AS, MK and TI revised and edited the manuscript. All the authors read and approved the final manuscript.

Data availability statement

All data and materials are available upon request from the authors.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. Dose-dependent expression of hHBegf (DT-R) and biochemical analyses of serum and urine in kidney- and liver-injury models

Table S1. Levels of markers for renal function in patients with several renal diseases including Dent disease

Table S2. Simple correlations of urinary FABP1 levels with biochemical variables