Proximal Tubular Cannabinoid-1 Receptor Regulates Obesity-Induced CKD

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ABSTRACT

Obesity-related structural and functional changes in the kidney develop early in the course of obesity and occur independently of hypertension, diabetes, and dyslipidemia. Activating the renal cannabinoid-1 receptor (CB_1R) induces nephropathy, whereas CB_1R blockade improves kidney function. Whether these effects are mediated *via* a specific cell type within the kidney remains unknown. Here, we show that specific deletion of CB_1R in the renal proximal tubule cells did not protect the mice from obesity, but markedly attenuated the obesity-induced lipid accumulation in the kidney and renal dysfunction, injury, inflammation, and fibrosis. These effects associated with increased activation of liver kinase B1 and the energy sensor AMP-activated protein kinase, as well as enhanced fatty acid β-oxidation. Collectively, these findings indicate that renal proximal tubule cell CB_1R contributes to the pathogenesis of obesity-induced renal lipotoxicity and nephropathy by regulating the liver kinase B1/AMP-activated protein kinase signaling pathway.

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The prevalence of obesity and its comorbidities has risen dramatically within the past two decades. Recently, more attention has been devoted to obesity-associated renal structural and functional changes, which develop early in the course of obesity.[1–3] In fact, obese individuals have a greater risk to develop ESRD,[4] especially in combination with diabetes and hypertension, which account for >70% of ESRD.[5,6] Obesity induces hemodynamic and morphologic changes in the kidney,[7] which together with renal inflammation[8] and oxidative stress,[9] may lead to reduced renal function and ultimately, glomerulosclerosis and tubulointerstitial fibrosis.[2,7,10,11] Although multiple metabolic factors have been proposed to contribute to obesity-induced nephropathy, the underlying signaling mechanisms are not completely understood.

Endocannabinoids (eCBs) are endogenous lipid ligands that interact with the cannabinoid-1 receptor (CB_1R), abundantly expressed in the brain and periphery, including the kidney.[12] Accumulating evidence shows the potential role of the eCB system in various renal pathologies.[13] CB_1Rs are expressed in podocytes,[14–16] mesangial cells,[17] and particularly in the renal proximal tubular cells (RPTCs),[12,18,19] and their blockade with CB_1R antagonists improves renal function and reduces albuminuria and glomerular lesions in obese and diabetic mouse models.[14,15,20–23] *In vitro*, the main eCB (arachidonoyl ethanolamide) increases RPTC hypertrophy, whereas CB_1R blockade reduces it,[19] and ameliorates palmitic acid-induced apoptosis of these cells.[18] However, most of these studies failed to determine whether the eCB system also plays a role in obesity-associated renal pathologies. If it does, is it mediated centrally, peripherally, or *via* a specific cell type within the kidney?

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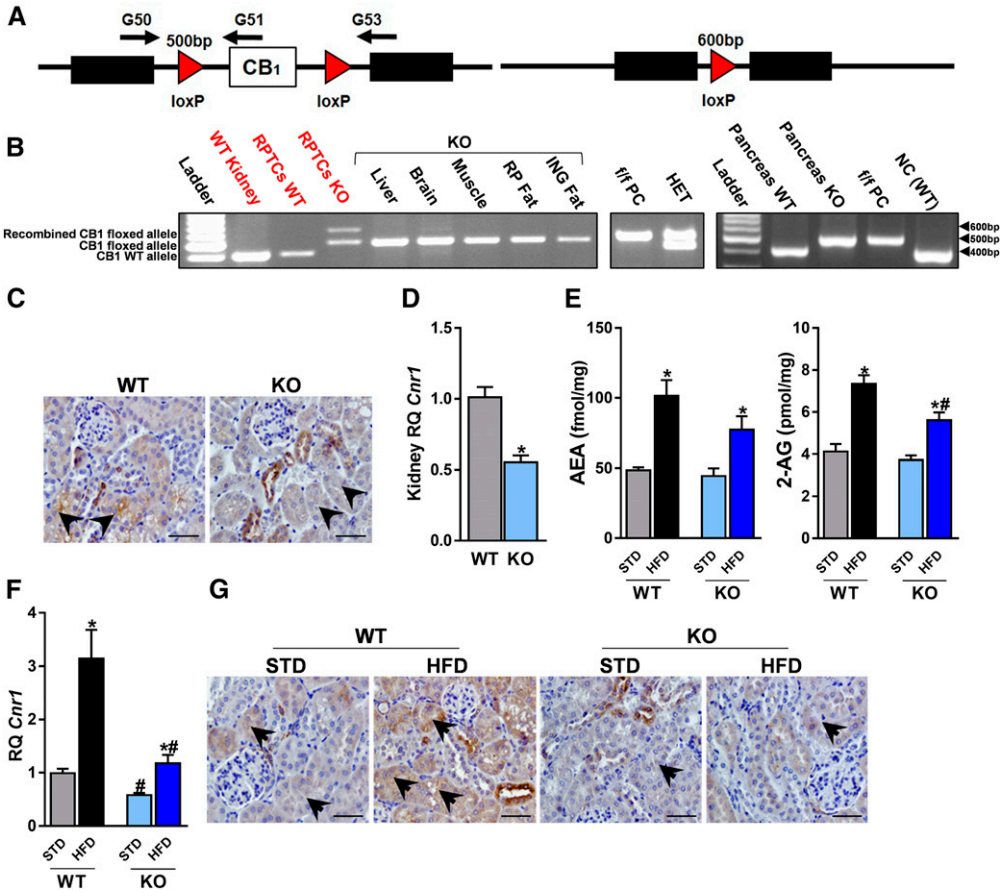

Figure 1. High-fat diet-induced obesity results in increased renal eCB tone. CB_1R gene locus containing flox sites (red triangles) with a forward G50 primer and reverse G51 and G53 primers (black arrows). *Cre* recombination results in a 600 bp product, whereas no recombination results in a 500 bp product (A). *Cre* recombination is shown only in RPTCs isolated from RPTC-$CB_1R^{-/-}$ mice and not in cells extracted from wild-type mice or in tissues such as liver, brain, muscle, inguinal, and retroperitoneal fat pads and pancreas. (B). Loss of CB_1R protein expression specifically in RPTCs (black arrows), with its normal expression in the distal tubule and glomerulus in RPTC-$CB_1R^{-/-}$ mice compared with their wild-type littermate control animals (C). Whole renal CB_1R mRNA expression levels were greatly reduced in RPTC-$CB_1R^{-/-}$ mice compared with their wild-type littermate controls (D). HFD feeding induced a parallel increase in renal arachidonoyl ethanolamide and 2-arachidonoylglycerol levels in both mouse strains (E). CB_1R mRNA expression levels were greatly reduced in RPTC-$CB_1R^{-/-}$ mice under both diets (F). The HFD-induced increase in CB_1R protein expression levels in wild-type control animals was limited to the RPTCs (black arrows) and not to distal tubules or glomerulus (G). Note the absence of HFD-induced upregulation in CB_1R protein expression in the RPTC-$CB_1R^{-/-}$ mice. Scale bar, 20 μm. Data represent the mean±SEM from 8 to 10 mice per group. *$P<0.05$ relative to animals on STD of the same genotype; $^{\#}P<0.05$ relative to wild-type animals on the same diet. HET, heterozygous; NC, negative control; PC, positive control.

To address these questions, we focused on the RPTCs responsible for active renal reabsorption by a mechanism requiring a large amount of energy derived from fatty acid β-oxidation.[24] RPTCs are particularly sensitive to the accumulation of lipids, an effect called lipotoxicity, which is regulated by the cellular energy and redox sensor, AMP-activated protein kinase (AMPK).[25,26] AMPK inactivates acetyl-CoA carboxylase (ACC) by its phosphorylation, consequently reducing malonyl CoA, leading to increased activity of carnitine palmitoyl transferase-1. This increases the translocation of fatty acids across the mitochondrial membrane, thereby increasing fatty acid β-oxidation.[27] Our findings suggest that manipulating RPTC-CB_1R may be a novel therapeutic approach for treating obesity-induced nephropathy.

RESULTS

Generating RPTC-Specific CB_1R-Null Mice

To explore the role of CB_1R on the RPTCs in mediating obesity-induced renal dysfunction, we generated RPTC-specific CB_1R-null mice (RPTC-$CB_1R^{-/-}$) by crossing CB_1R-floxed mice with transgenic iL1-sglt2-Cre mice,[28] specifically expressing *Cre* recombinase in the brush border membrane of

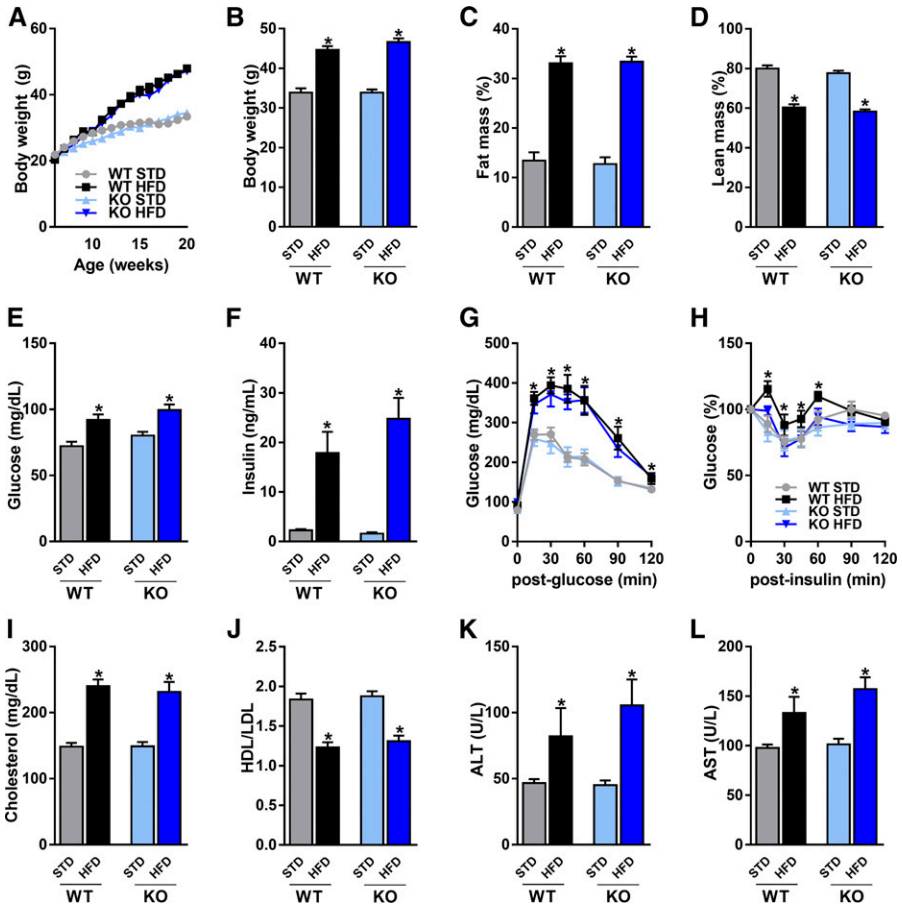

Figure 2. RPTC-$CB_1R^{-/-}$ mice are sensitive to HFD-induced obesity and have similar metabolic abnormalities. RPTC-$CB_1R^{-/-}$ mice gained a similar body weight (A and B) and displayed equal fat (C) and lean (D) body masses as their wild-type littermate controls. Loss of CB_1R in RPTCs did not alter glucose homeostasis, as measured by fasting blood glucose (E), serum insulin levels (F), and the glucose tolerance test (G). An insulin sensitivity test revealed a significant improvement in the null mice on HFD (H). The absence of CB_1R in RPTCs did not change the lipid profile, as determined by the serum cholesterol levels (I) as well as the HDL/LDL cholesterol ratio (J). RPTC-$CB_1R^{-/-}$ mice exhibited a similar liver dysfunction on HFD, as measured by the serum levels of ALT (K) and AST (L). Data represent the mean±SEM from 8 to 10 mice per group. *$P<0.05$ relative to animals on STD of the same genotype.

the *S1* segment of the proximal tubule. Genomic DNA analysis revealed that CB_1R gene deletion occurred specifically in RPTCs isolated from mouse kidneys, whereas its deletion was excluded from the liver, brain, muscle, fat pads, and pancreas (Figure 1, A and B). Additionally, RPTCs were completely devoid of CB_1R protein expression, whereas its expression pattern remained normal in the distal tubular cells and glomerulus (Figure 1C). Likewise, the mRNA expression of CB_1R in the whole kidney was significantly reduced (Figure 1D), indicating its substantial expression in RPTCs.[12]

RPTC-$CB_1R^{-/-}$ Mice Are Sensitive to Obesity Induced by High-Fat Diet

When maintained on a high-fat diet (HFD) for 14 weeks, an equal elevation in renal arachidonoyl ethanolamide and 2-arachidonoylglycerol levels was documented in both mouse strains (Figure 1E). Importantly, the renal expression of CB_1R was significantly reduced in the RPTC-$CB_1R^{-/-}$ mice (Figure 1, F and G). However, this did not affect the susceptibility of the mice to develop obesity because both the RPTC-$CB_1R^{-/-}$ mice and their wild-type littermates developed a similar degree of obesity (Figure 2, A and B), associated with increased fat and reduced lean body mass (Figure 2, C and D). Both obese mouse strains showed equal disturbances in glucose homeostasis (Figure 2, E and G). Surprisingly, the obese knockouts were more sensitive to insulin (Figure 2H). A similar degree of hypercholesterolemia, a low HDL/LDL ratio, and liver injury were measured in both strains (Figure 2, I–L). An exacerbated metabolic phenotype was found when the mice maintained on HFD for 43 weeks, although slight metabolic improvements were noted in RPTC-$CB_1R^{-/-}$ mice (Supplemental Figure 1, A–H). No differences in BP were found between wild-type mice on standard diet (STD) and HFD, as well as between the two mouse strains (Supplemental Figure 2).

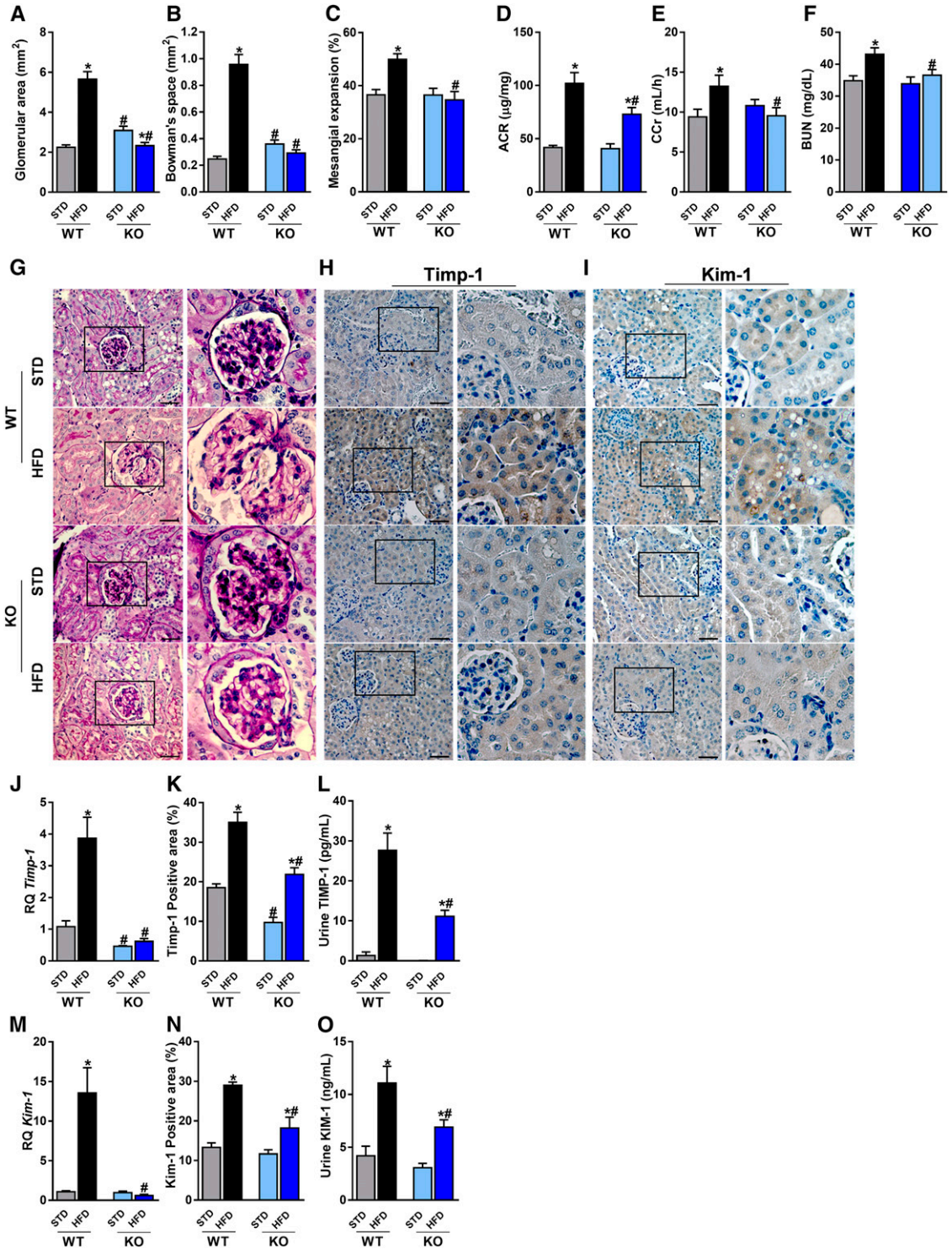

Figure 3. RPTC-$CB_1R^{-/-}$ mice are protected from obesity-induced morphologic changes in the kidney, renal dysfunction, and injury. The HFD-induced morphologic changes in the kidney, such as glomerular enlargement (A and G), an increased Bowman's space area (B and G), and a mesangial expansion (C and G) were attenuated in obese RPTC-$CB_1R^{-/-}$ mice. Similarly, the HFD-induced kidney dysfunction, as manifested by the increased albumin-to-creatinine ratio (D), creatinine clearance (E), and BUN (F), was markedly attenuated in the obese RPTC-$CB_1R^{-/-}$ mice. The HFD-induced upregulation in renal mRNA and protein expression levels as well as the urine excretion levels of the kidney injury markers TIMP-1 (H and J–L), and KIM-1 (I and M–O) were attenuated or normalized in RPTC-$CB_1R^{-/-}$ mice on the same diet. Scale bar, 20 μm. Original magnification, ×3 in G, H, and I. Data represent the mean±SEM from 8 to 10 mice per group. *$P<0.05$ relative to animals on STD of the same genotype; $^{\#}P<0.05$ relative to wild-type animals on the same diet.

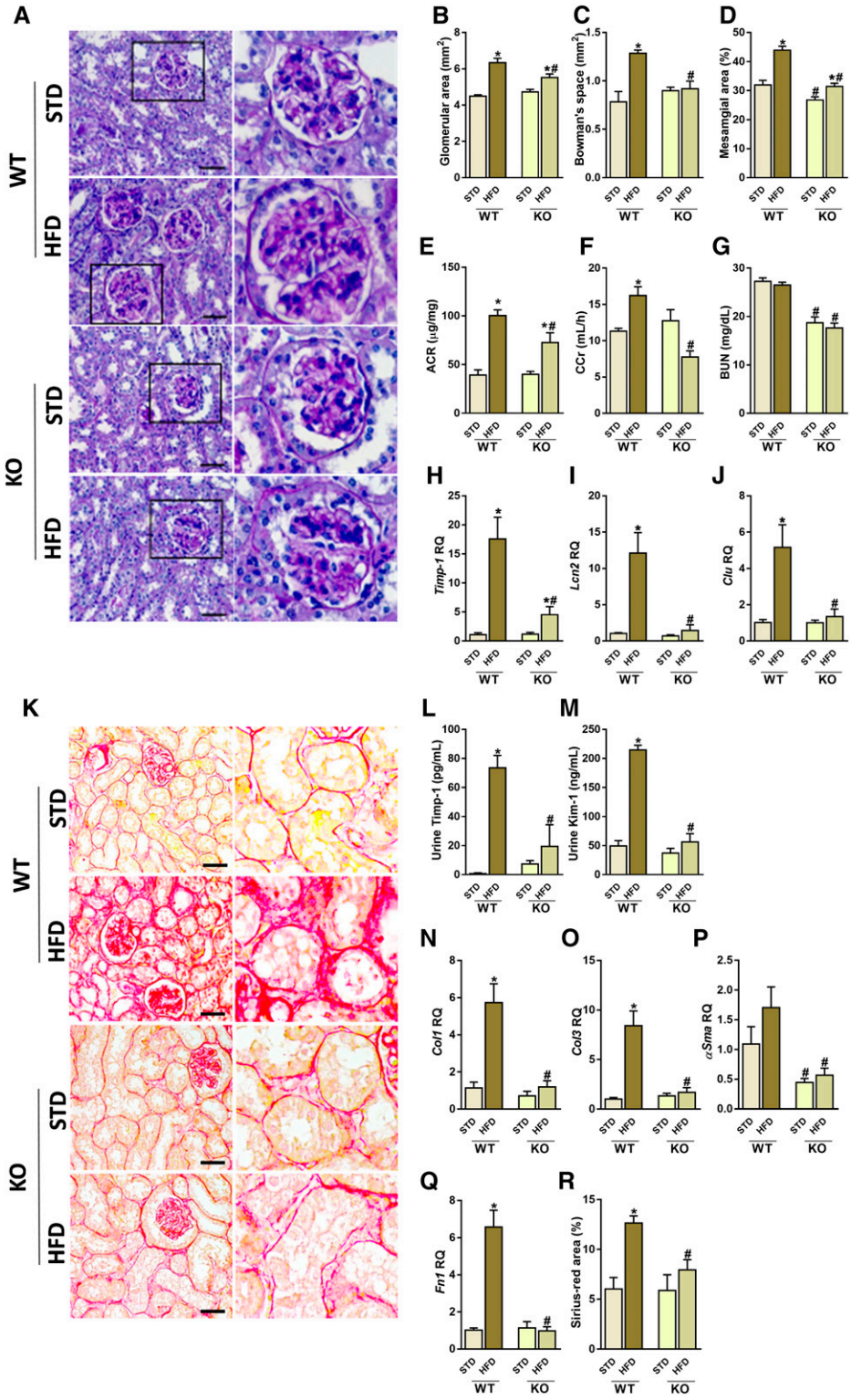

Figure 4. RPTC-CB$_1$R$^{-/-}$ mice are protected from obesity-induced morphologic changes in the kidney, renal dysfunction and injury even after a very prolonged (43 weeks) duration of HFD feeding. The HFD-induced morphologic changes in the kidney, such as glomerular enlargement (A and B), an increased Bowman's space area (A and C), and mesangial expansion (A and D) were attenuated in obese RPTC-CB$_1$R$^{-/-}$ mice. The HFD-induced kidney dysfunction, as manifested by the increased albumin-to-creatinine ratio (E), creatinine clearance (F), and increased BUN (G) was significantly attenuated in the obese RPTC-CB$_1$R$^{-/-}$ mice. The HFD-induced upregulation in the renal mRNA expression levels of the kidney injury markers *Timp-1* (H), *Lcn2* (I), and *Clu* (J), as well as in the urinary

Metabolically, both obese mouse strains exhibited a similar alteration in the basal metabolic rate compared with those on STD (Supplemental Figure 3, A–D). Combined with a similar activity profile and food and water intake (Supplemental Figure 3, E–H), these findings suggest that CB_1R in RPTCs does not contribute to obesity and its associated comorbidities in mice.

RPTC-$CB_1R^{-/-}$ Mice Are Protected from Obesity-Induced Kidney Injury

Enlarged glomerular and Bowman's space (Figure 3, A, B, and G) areas, increased mesangial expansion (Figure 3, C and G), as well as markedly elevated albumin-to-creatinine ratio (Figure 3D), creatinine clearance (Figure 3E), and BUN levels (Figure 3F) were found in obese wild-type control mice. These changes were associated with marked elevations in several kidney injury markers (Figure 3, H–O, Supplemental Figure 4). All of these effects were normalized/attenuated in the obese RPTC-$CB_1R^{-/-}$ animals. Despite increased obesity after a prolonged HFD feeding (43 weeks), little further progression of renal damage was observed in obese wild-type mice (Figure 4, A–J, L, and M). Kidney-to-body weight ratios were reduced in both mouse strains on HFD, although significantly less reduction was documented in the RPTC-$CB_1R^{-/-}$ mice, probably attributable to their larger kidneys on baseline conditions (Supplemental Figure 5).

RPTC-$CB_1R^{-/-}$ Mice Are Protected from Obesity-Induced Renal Inflammation and Tubulointerstitial Fibrosis

Next, measuring the renal expression levels of several proinflammatory (Figure 5, A–F) and profibrotic markers (Figure 5, G, H, and J–L) revealed marked increases in obese wild-type controls and normal expression in obese RPTC-$CB_1R^{-/-}$ mice. Moreover, quantifying renal fibrosis revealed a significant collagen deposition in wild-type animals on HFD, but not in RPTC-$CB_1R^{-/-}$ mice (Figure 5, I and M). A similar pattern was documented in wild-type mice fed HFD for 43 weeks (Figure 4, K and N–R). Enhanced renal expression and localization of the proliferative and macrophage markers, Ki67 and F4/80, respectively, was only found in obese wild-type mice (Supplemental Figure 6), suggesting a key role of RPTC-CB_1R in mediating HFD-induced renal inflammation, macrophage infiltration, and tubulointerstitial fibrosis.

CB_1R Deletion/Blockade Ameliorates Lipid Accumulation in RPTCs *via* an AMPK/ACC Signaling Pathway

A significant feature of obesity-related renal dysfunction is the accumulation of lipid droplets in the kidney. In accordance with others,[26,29] quantifying vacuolated proximal tubules in HFD-fed mice revealed a high accumulation of lipid droplets in wild-type mice, but not in the obese RPTC-$CB_1R^{-/-}$ animals, after either 14 (Figure 6, A and D) or 43 (Supplemental Figure 7, A and C) weeks on HFD. Molecularly, CB_1R is a key regulator of AMPK in different tissues,[30–32] and modulating this signaling pathway affects both fatty acid β-oxidation and lipogenesis in the kidney.[25,26,29] Likewise, we found a significant reduction in the activated/phosphorylated form of AMPK after 7 days (Figure 6, G and I), 14 weeks (Figure 6, B and E), and even 43 weeks (Supplemental Figure 7, B and D) on HFD. Moreover, the expression of the nonactivated/phosphorylated form of its downstream substrate, ACC, was dramatically downregulated under the same conditions (14 weeks: Figure 6, C and F; 7 days: Figure 6, H and J), an effect that may promote lipid accumulation in the kidney. Importantly, these effects were completely absent in the obese RPTC-$CB_1R^{-/-}$ mice, as well as in obese wild-type mice treated with the peripherally restricted CB_1R antagonist, JD5037 (Figure 6, G–J).[33] Increasing the *in vivo* production of eCBs by JZL195[34] resulted in a significant reduction in the phosphorylated AMPK and ACC in the kidney (Supplemental Figure 8).

Exposing RPTCs to a mixture of oleate/palmitate for 24 hours *in vitro* greatly elevated the levels of 2-arachidonoylglycerol and its endogenous precursor and degrading ligand, AA (Figure 7A), and the expression of CB_1R (Figure 7, B and C). These changes were associated with elevated expression levels of several proinflammatory cytokines (Figure 7D). Likewise, directly activating CB_1R by the synthetic agonist arachidonyl-2'-chloroethylamide (ACEA) upregulated these markers (Figure 7E). Both effects were completely normalized by pretreating the cells with JD5037. In accordance with the *in vivo* findings, we also found that JD5037 completely reversed the oleate/palmitate– and ACEA-induced downregulation in both the pAMPK/AMPK and pACC/ACC ratios (Figure 7, F, H, I, K, and L), and consequently reduced the accumulation of fat droplets in the cells (Figure 7M).

Role of the Gi-PKA-LKB1 Axis in Activating AMPK by CB_1R Deletion/Blockade in RPTCs

Two upstream kinases, the tumor suppressor liver kinase B1 (LKB1) and the Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ), modulate AMPK activity by inducing its phosphorylation.[35,36] *In vivo*, HFD reduced the phosphorylated (activated) form of LKB1 on Ser428 residue in the kidney, whereas genetic deletion or pharmacologic blockade of CB_1R ameliorated it (Figure 6, K–N, Supplemental Figure 7, E and F). Similarly,

excretion levels of TIMP-1 (L) and KIM-1 (M) were normalized in RPTC-$CB_1R^{-/-}$ mice on the same diet. Similarly, the HFD-induced renal fibrosis was attenuated in RPTC-$CB_1R^{-/-}$ mice, as ascertained by measuring the renal mRNA expression levels of the profibrotic markers *Col1* (N), *Col3* (O), α*Sma* (P), and *Fn1* (Q), as well as by quantifying collagen deposition by Sirius Red staining (K and R). Scale bar, 20 μm. Original magnification, ×3 in A and K. Data represent the mean±SEM from 5 to 10 mice per group. *$P<0.05$ relative to animals on STD of the same genotype; $^{\#}P<0.05$ relative to wild-type animals on the same diet.

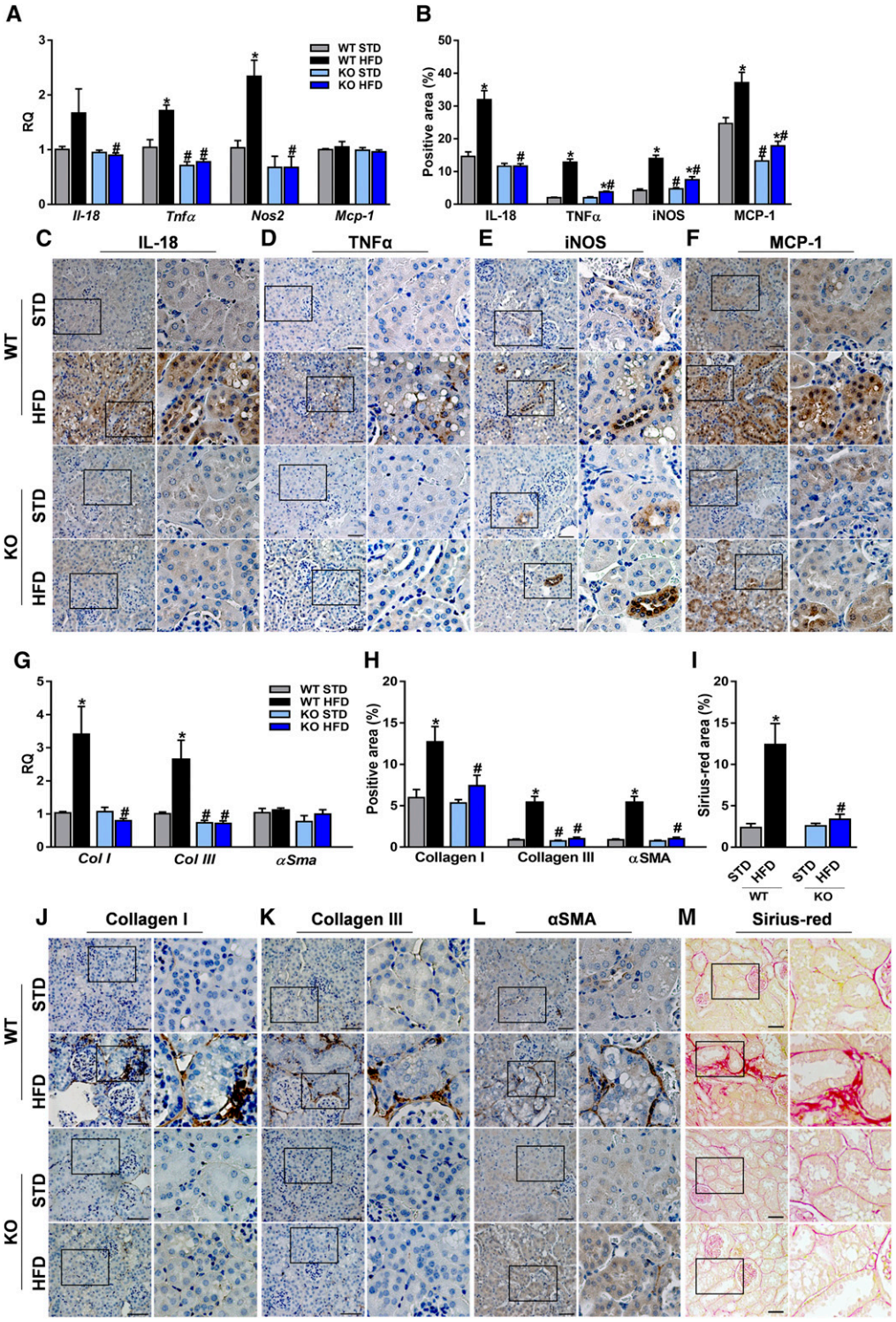

Figure 5. RPTC-$CB_1R^{-/-}$ mice are protected from obesity-induced tubulointerstitial inflammation and fibrosis. The HFD-induced upregulation in the renal mRNA and the protein expression levels of the inflammatory markers IL-18 (A, B, and C), TNFα (A, B, and D), iNOS (A, B, and E), and MCP-1 (A, B, and F) were attenuated or normalized in the RPTC-$CB_1R^{-/-}$ mice on the same diet. The HFD-induced upregulation in the renal mRNA and protein expression levels of the fibrogenic markers collagen-1 (G, H, and J), collagen-3 (G, H, and K), and αSMA (G, H, and L) were attenuated or normalized in the RPTC-$CB_1R^{-/-}$ mice on the same diet. Similarly, an elevated collagen deposition, measured by Sirius Red staining, was evident in the HFD-fed wild-type controls compared with the RPTC-$CB_1R^{-/-}$ mice on the same diet (I and M). Scale bar, 20 μm. Original magnification, $\times$3 for all inserts. Data represent the mean$\pm$SEM from 8 to 10 mice per group. *$P<0.05$ relative to animals on STD of the same genotype; $^{\#}P<0.05$ relative to wild-type animals on the same diet.

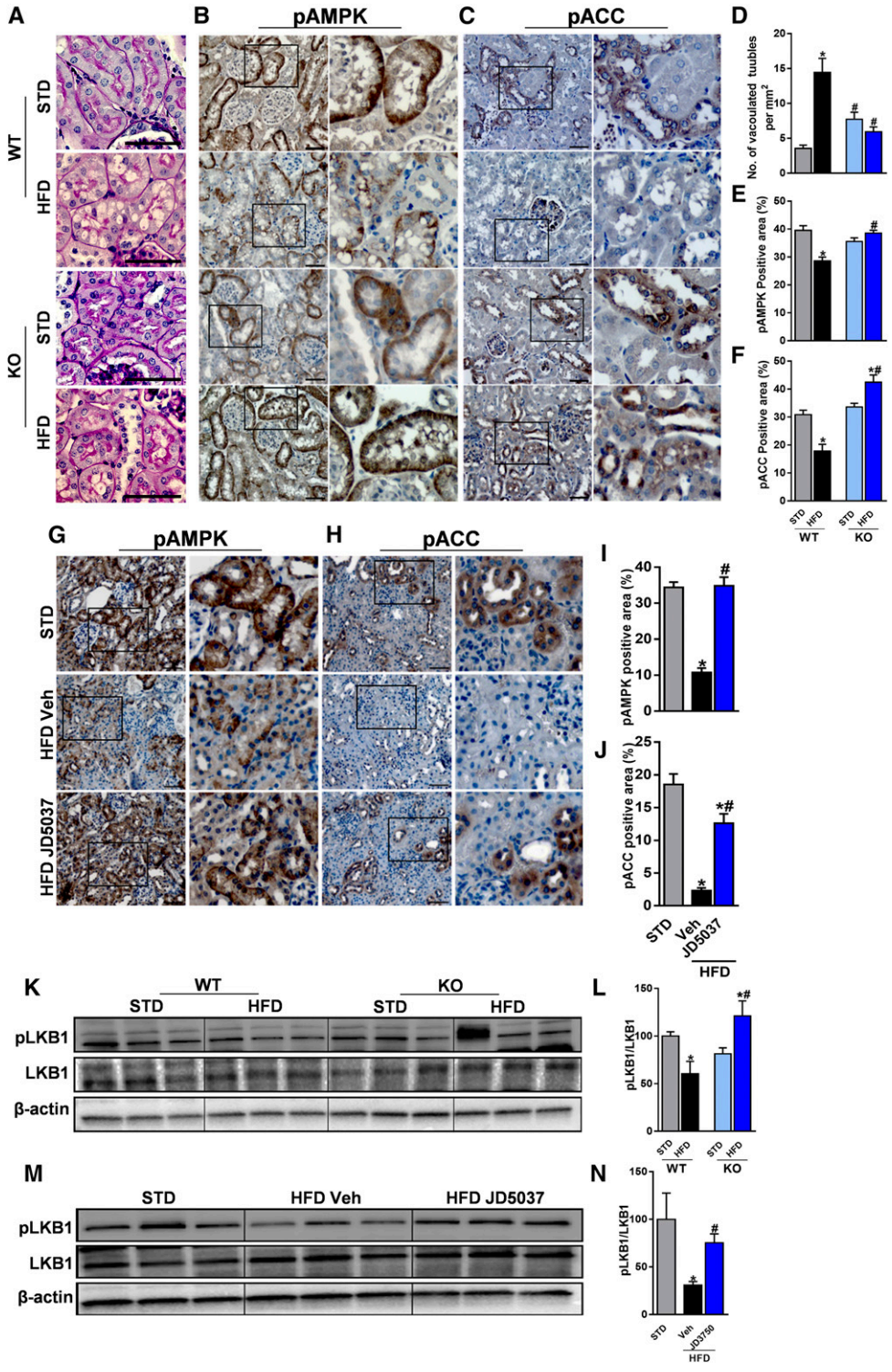

Figure 6. Genetic deletion or pharmacologic blockade of CB_1R ameliorates lipid accumulation in RPTCs *via* a LKB1/AMPK/ACC signaling pathway. A high accumulation of lipid droplets was found in obese wild-type mice, and not in RPTC-$CB_1R^{-/-}$ animals (A and D). Significant reductions in the expression of the activated/phosphorylated form of AMPK after both long (14 weeks; B and E) and short (7 days; G and I) periods of HFD feeding and in the nonactivated/phosphorylated form of ACC (14 weeks: C and F; 7 days: H and J) as well as in the activated/phosphorylated form of LKB1 (14 weeks: K and L; 7 days: M and N) were found in obese wild-type mice. Genetic deletion of CB_1R in RPTCs (B, C, E, F, K, and L) or the peripheral blockade of CB_1R by JD5037 (3 mg/kg, administered orally) (G–J, M, and N) significantly normalized the expression levels of pLKB1, pAMPK, and pACC. Scale bar, 20 μm. Original magnification, ×3 for all inserts. Data represent the mean±SEM from 8 to 10 mice per group. *$P<0.05$ relative to animals on STD of the same genotype; $^{\#}P<0.05$ relative to wild-type animals on the same diet.

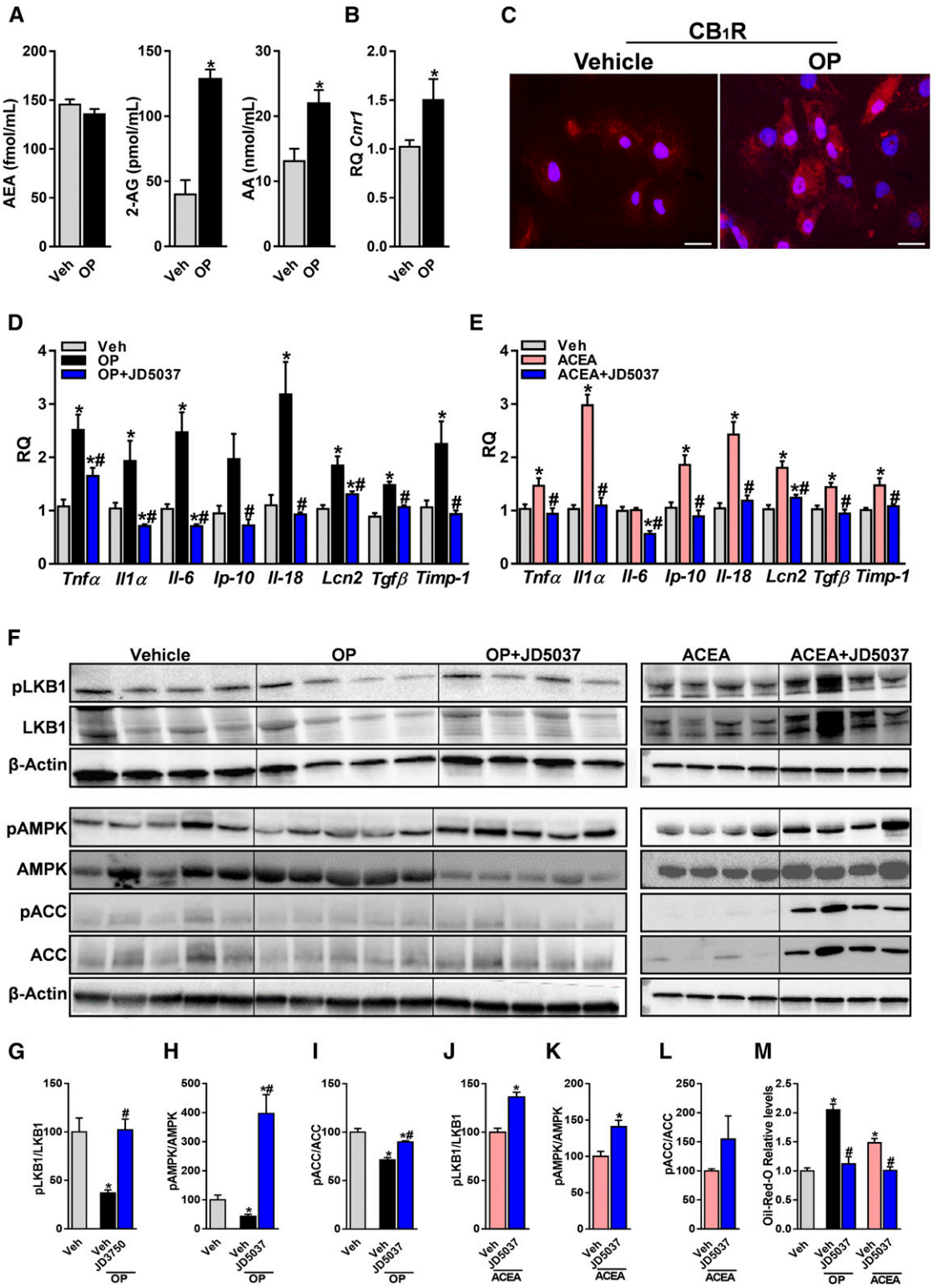

Figure 7. Peripheral CB_1R blockade reverses inflammation and lipid accumulation in RPTCs *via* AMPK signaling. Increased activity of the eCB system was reflected by the elevated levels of 2-arachidonoylglycerol and its endogenous precursor and degraded ligand AA, but not arachidonoyl ethanolamide (A) as well as the upregulation of mRNA (B) and the protein (C) expression levels of CB_1R in HK-2 cells exposed to 0.5 mM oleate/palmitate for 24 hours. This elevation was associated with upregulating the mRNA expression levels of several kidney injury and inflammatory markers, such as *Tnfα*, *Il-1α*, *Il-6*, *Ip-10*, *Il-18*, *Lcn2*, *Tgf-β*, and *Timp-1* (D). Likewise, a direct activation of CB_1R by the synthetic agonist ACEA resulted in similar upregulation of these genes (E). Both effects were completely

JD5037 completely prevented the oleate/palmitate– and ACEA-induced downregulation in pLKB1 on RPTCs *in vitro* (Figure 7, F, G, and J). Conversely, a specific blocker of CaMKKβ (STO-609, 1 μg/ml) failed to inhibit the activation of AMPK by JD5037 in the presence of oleate/palmitate (Supplemental Figure 9).

Next, we tested the role of G$\alpha_{i/o}$-dependent protein kinase A (PKA), which was shown to regulate the activity of LKB1 elsewhere.[37] Adding 100 ng/ml of pertussis toxin (a G$\alpha_{i/o}$ blocker) to RPTCs treated with ACEA caused a significant increase in phosphorylation of LKB1, AMPK, and ACC compared with the effect of ACEA alone (Figure 8, A and C), suggesting Gi protein involvement. Moreover, the increased phosphorylation by JD5037 with oleate/palmitate was abolished by pretreating RPTCs with 500 nM H-89 (a PKA blocker; Figure 8, B and D). Collectively, these findings imply that CB_1R modulates obesity-induced lipotoxicity in the RPTCs *via* regulating the LKB1/AMPK/ACC signaling pathway.

CB_1R Deletion/Blockade Increases Fatty Acid β-Oxidation in RPTCs

Next, we found that the HFD-induced reduction in the renal expression of *Ppar*α, *Slc27a*, and *Fabp1*, which encode proteins important for utilizing fatty acids in the mitochondria, is ameliorated in RPTC-$CB_1R^{-/-}$ mice (Figure 8E). Similarly, JD5037 completely reversed the oleate/palmitate– or ACEA-induced reduction in the expression of these genes in RPTCs (Figure 8, F and G). Accordingly, the basal and maximal respiratory rates were markedly elevated (Figure 8, H–J), and intracellular ATP production was vastly increased (Figure 8K) in JD5037-treated RPTCs. Taken together, these findings suggest that deleting/blocking CB_1R in RPTCs utilizes fatty acids by β-oxidation rather than by lipogenesis (Figure 8L), and protects the kidney from obesity-induced dysfunction and injury.

DISCUSSION

Obesity-related renal dysfunction develops early in the course of obesity, justifying the search for novel regulators that could then be targeted for therapy. Overactivation of the eCB/CB_1R system in obesity promotes tubulointerstitial lesions and glomerulopathy; however, the exact underlying molecular mechanism is not fully understood. This study demonstrates the role of proximal tubule CB_1R in renal lipotoxicity and consequently, in obesity-induced renal inflammation, fibrosis, and injury by governing the LKB1/AMPK/ACC signaling pathway. Hence, manipulating RPTC-CB_1R should be considered as a therapeutic approach for treating obesity-induced nephropathy.

Although the eCB/CB_1R system was shown to regulate renal hemodynamics, inflammation, and fibrogenesis during pathologic conditions,[13] it was not determined whether these abnormalities are mediated *via* CB_1R on a specific cell type within the kidney. By developing a transgenic mouse line, which specifically lacks the CB_1R gene in RPTCs (driven by SGLT2-Cre recombination), we could answer this question. The absence of the SGLT2 gene and protein in extrarenal organs was reported by others.[28,38–40] However, a recent report demonstrated that SGLT2 protein is expressed in glucagon-expressing α cells of the human pancreas.[41] Because the expression of CB_1R in pancreatic α cells is still controversial,[42–45] we cannot exclude the possible absence of its protein in these cells in RPTC-$CB_1R^{-/-}$ mice. Nevertheless, we did not detect a Cre-Lox recombination in pancreas collected from the null mice, and the contribution of such a loss (if present) to the metabolic and/or renal phenotypes of these mice may be minimal because most mouse and human α cells do not express SGLT2 and CB_1R.[46]

Global CB_1R knockout mice[47] and animals with a selective genetic deletion[48] or downregulation of CB_1R in CaMKKa[49] or hypothalamic neurons[50] are lean and resistant to HFD-induced obesity, suggesting that hypothalamic and/or peripheral sympathetic CB_1R is required for developing obesity. However, this does not negate the crucial role of CB_1R in peripheral organs during obesity. Indeed, mice lacking hepatic CB_1R develop obesity on HFD similarly to their littermates; however, they are protected from obesity-induced hepatic steatosis.[51] Likewise, both mouse strains used here become obese and metabolically impaired; however, RPTC-$CB_1R^{-/-}$ mice are largely protected from the deleterious effect of a prolonged fatty diet on the kidneys. These findings indicate that activating RPTC-CB_1Rs is crucial for producing nephropathy during obesity. Nevertheless, our findings do not exclude the possibility that other intrarenal or extrarenal CB_1Rs, such as CB_1R on glomerular podocytes, which contribute to glomerular dysfunction and damage in type 2 diabetic nephropathy,[52] may also contribute to nephropathy. In addition, the slight improvements in some of the metabolic parameters after a very prolonged HFD feeding in RPTC-$CB_1R^{-/-}$ mice are possibly related to an undefined role of CB_1R in RPTCs in modulating these parameters.

Independently of the metabolic syndrome, obesity induces structural changes in the kidney, leading to reduced renal function.[1–4] Our findings in obese wild-type mice appear to

normalized by pretreating the cells with the peripherally restricted CB_1R inverse agonist JD5037 (100 nM). Similarly, JD5037 reversed the oleate/palmitate–induced downregulation in the phosphorylated forms of LKB1, AMPK, and ACC (F–I), as well as increased the phosphorylation of these proteins despite the presence of ACEA (F, and J–L). Pretreating HK-2 cells with JD5037 also reversed the accumulation of fat droplets in cells with oleate/palmitate or ACEA (M). Scale bar, 20 μm. Data represent the mean±SEM from three independent experiments. *$P<0.05$ relative to vehicle-treated cells under normal conditions; #$P<0.05$ relative to vehicle-treated cells under oleate/palmitate conditions or ACEA treatment.

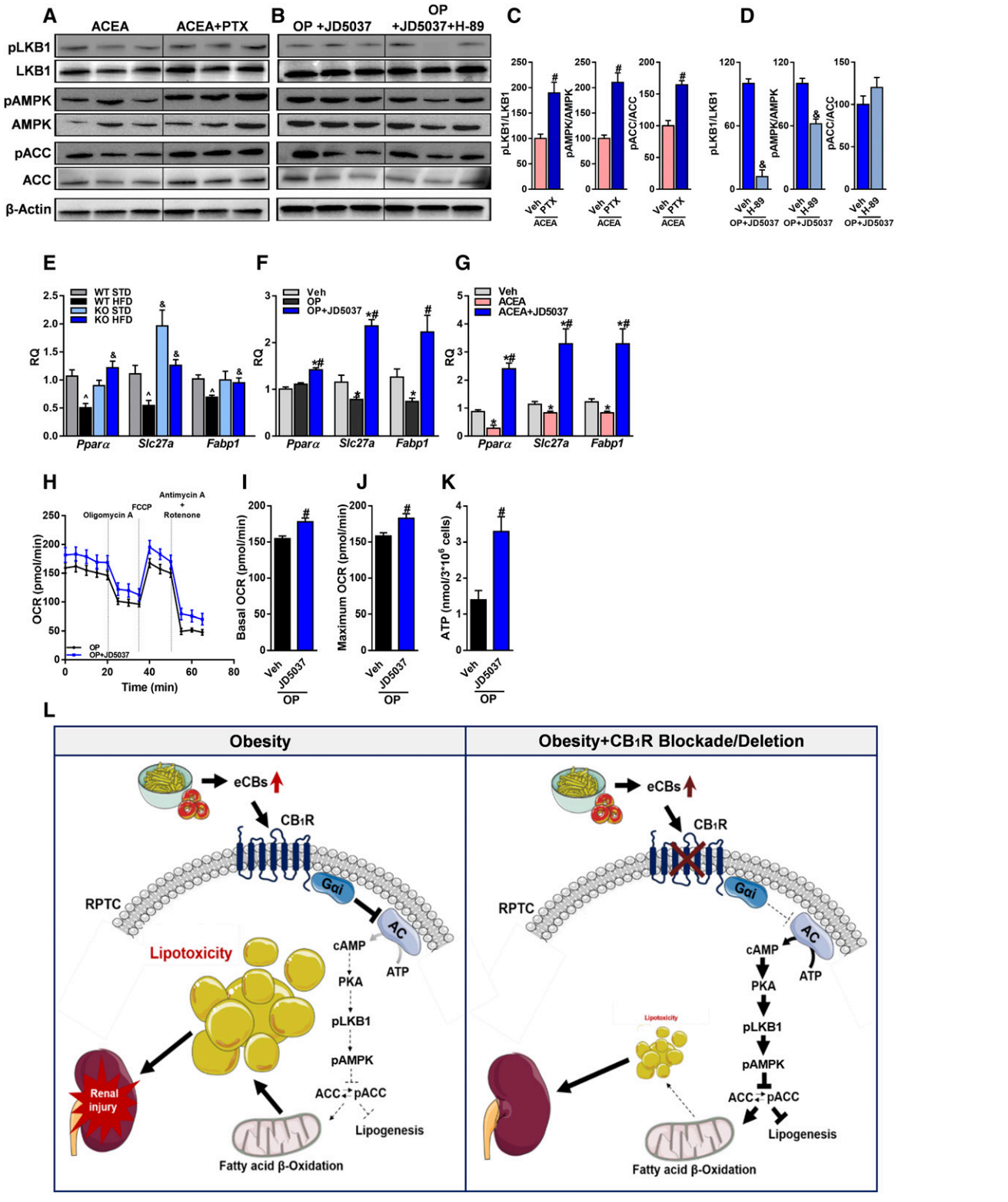

Figure 8. CB_1R modulates lipogenesis or fatty acid β-oxidation in RPTCs *via* the Gi-PKA axis. Pretreating HK-2 cells with 100 ng/ml pertussis toxin (PTX; a $G\alpha_{i/o}$ blocker) with the CB_1R agonist ACEA significantly increased the phosphorylation of LKB1, AMPK, and ACC (A and C). JD5037 loses the ability to increase the phosphorylation of these proteins while incubating HK-2 cells with 500 nM H-89 (a PKA inhibitor) (B and D). Reduced mRNA expression levels of *Pparα*, *Slc27a*, and *Fabp1* were documented in HFD-induced obese wild-type mice (E) as well as in HK-2 cells treated with oleate/palmitate (F) or ACEA (G). These reductions were normalized by either genetic ablation or pharmacologic blockade of CB_1R in RPTCs. Increased basal and maximal respiratory rates (H–J) as well as

be consistent with this notion. Remarkably, obese RPTC-$CB_1R^{-/-}$ mice are not only resistant to the HFD-induced structural and functional changes in the kidney; they also do not develop an inflammatory response, and are resilient to developing renal fibrosis even after 43 weeks on HFD. Moreover, the fact that both mouse strains have similar levels of eCBs in the kidney but a significant difference in renal CB_1R expression, indicates the pivotal role of CB_1R in RPTCs for maintaining renal homeostasis and function.

Surprisingly, only a minor progression in renal damage was observed in wild-type mice consuming approximately 30% saturated fat for 43 weeks. Similar findings were also reported by others,[53] demonstrating that HFD-induced obesity (24 weeks) aggravates age-dependent kidney dysfunction, with no major increase in kidney injury after 40 weeks of feeding. It is reasonable to assume that higher saturated fat content may promote renal dysfunction over time. However, HFD may enhance kidney dysfunction of different etiologies independently of hypertension,[54] including intrauterine growth restriction,[55] type 1 diabetic nephropathy,[56] aging,[53] IgA GN,[57] and uninephrectomy.[58] Moreover, under certain clinical conditions, such as diabetic nephropathy,[14,20,59] IgA nephropathy,[60] and acute interstitial fibrosis,[60] there is increased activation of the renal eCB/CB_1R system. Although none of these studies determined whether it enhances susceptibility to HFD-induced renal injury, it is reasonable to suggest that superimposing HFD under these conditions most likely would exacerbate kidney damage. Indeed, genetic analyses implicate lipotoxicity in the susceptibility to type 2 diabetic nephropathy.[61] Because evidence for the role of the eCB/CB_1R system in diabetic nephropathy has been reported in humans and murine models,[15,52,62] we also may speculate that HFD would probably augment type 2 diabetes–induced kidney damage by enhanced lipotoxicity.

HFD-induced obesity results in the excessive accumulation of lipid vacuoles in RPTCs. Similarly, exposing RPTCs to oleate/palmitate resulted in increased intracellular fat accumulation associated with upregulating eCBs and CB_1R. A similar effect was also found by directly activating CB_1R in RPTCs, suggesting that the eCB system plays a key role in mediating lipid metabolism in RPTCs. These findings are in accordance with others, who reported increased RPTC apoptosis in the presence of palmitic acid, an effect that depended on CB_1R-induced stimulation of the endoplasmic reticulum stress response.[18] Altered lipid metabolism and deposition have also been reported in obese humans with CKD.[63] Intracellular lipid accumulation is normally regulated by a balance between the influx of fatty acids, their synthesis, and breakdown, as well as their oxidation and efflux.[26,63] Previous studies showed that this process and its related kidney damage are associated with reduced activity of AMPK in the kidney.[25,26,29] AMPK, abundantly expressed in the kidney, restores energy balance by promoting energy-generating pathways. Together with ACC, regulation of fatty acid β-oxidation and synthesis occurs. Our study clearly supports these observations, and, for the first time, highlights the pivotal role of blocking or deleting CB_1R in enhancing fatty acid β-oxidation rather than promoting lipid accumulation in RPTCs.

As reported in hepatocytes,[37] we show that activating AMPK by inhibiting CB_1R is controlled upstream by a Gi-PKA-LKB1 axis, without the involvement of CaMKKβ. This axis, identified here in RPTCs, shows that CB_1R, coupled with G proteins of the $G\alpha_{i/o}$,[64] triggers a signaling pathway that inhibits the activation of PKA, which directly phosphorylates LKB1, the upstream kinase of AMPK, responsible for its activity. The importance of LKB1 in modulating intracellular metabolism in our model is supported by a recent work demonstrating that its deletion in renal distal tubular cells alters fatty acid metabolism *via* AMPK signaling.[65]

Collectively, we demonstrated that lipid accumulation and reduced fatty acid β-oxidation in RPTCs, associated with obesity-induced renal abnormalities, are governed by a CB_1R-coupled $G\alpha_{i/o}$-PKA axis, which mediates the downstream activation of the LKB1/AMPK/ACC signaling pathway (Figure 8L). Thus, manipulating CB_1R specifically in the RPTCs may provide a novel therapeutic intervention for treating obesity-induced nephropathy.

CONCISE METHODS

Please see Supplemental Material for a detailed description of the methods.

Animals and Experimental Protocol

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Hebrew University. RPTC-$CB_1R^{-/-}$ mice were generated by crossing mice containing two loxP sites flanking the open reading frame of CB_1R with the iL1-sglt2-Cre line.[28] Six-week-old male RPTC-$CB_1R^{-/-}$ mice and their wild-type littermates were fed either STD or HFD for 14 or 43 weeks. Two additional mouse cohorts of C57Bl/6J mice were used: (*1*) mice on HFD treated with JD5037 (3 mg/kg, administered orally) or vehicle; and (*2*) mice treated with JZL195 (20 μg/g, administered intraperitoneally).

ATP production (K) were measured in HK-2 cells treated with JD5037 (100 nM) with oleate/palmitate. The suggested molecular mechanism by which CB_1R in RPTCs regulates renal lipotoxicity (L). *In vitro* data represent the mean±SEM from three independent experiments. $^{*}P<0.05$ relative to vehicle-treated cells under normal conditions; $^{\#}P<0.05$ relative to vehicle-treated cells under oleate/palmitate conditions or ACEA treatment; $^{\&}P<0.05$ relative to JD5037-treated cells under oleate/palmitate conditions. *In vivo* data represent the mean±SEM from 8 to 10 mice per group. $^{\wedge}P<0.05$ relative to animals on STD of the same genotype; $^{\&}P<0.05$ relative to wild-type animals on the same diet.

Cell Culture Experiments
Human immortalized RPTCs were cultured in REGM BulletKit medium at 37°C in a humidified atmosphere of 5% CO_2/95% air. Lipotoxicity was achieved by 0.5 mM oleate/palmitate (2:1). Activating CB_1R was done by ACEA (10 μM). Extracellular flux analysis was recorded by Seahorse XF24. ATP content was quantified by using a commercial kit.

eCB Measurements
eCBs were extracted, purified, and quantified in kidney and cell supernatant, as described previously.[66]

Multiparameter Metabolic Assessment
Mouse phenotypic analysis was assessed by the Promethion High-Definition Behavioral Phenotyping System, as described previously.[66]

Biochemistry
Serum and urinary biochemical analyses were determined by Cobas C-111. Urinary albumin, KIM-1, TIMP-1, LCN2, and serum insulin levels were measured by ELISAs. Glucose homeostasis tests were done as described previously.[66]

Genomic DNA Extraction
Mouse kidneys were harvested using a Percoll extraction and ultracentrifugation method. DNA was extracted and RT-PCR analysis for the CB_1R gene locus was performed as described previously.[67]

Western Blotting
Kidney and cell homogenates were blotted as described previously.[66]

Histopathology
Paraffin-embedded kidney sections (2–3 μm) were stained with periodic acid–Schiff and Sirius Red. The glomerular and Bowman's space cross-sectional areas and mesangial expansion were quantified using ZEN imaging software. Measuring tubular vacuole accumulation was done on periodic acid–Schiff-stained sections, as described previously.[26]

Immunohistochemistry and Immunofluorescence
RPTCs and paraffin-embedded kidney sections were used to determine protein expression using commercially available antibodies.

Real-Time PCR
Total mRNA from mouse kidney and RPTCs was extracted as described previously.[66] Primer information is described in Supplemental Tables 1 and 2.

Materials
JD5037[68] was purchased from Haoyuan Chemexpress Co., Ltd.

Statistical Analyses
Values are expressed as the mean±SEM. Unpaired two-tailed *t* test was used to determine differences between groups. Results in multiple groups and time-dependent variables were compared by ANOVA, followed by a Bonferroni test (GraphPad Prism v6 for Windows). Significance was set at $P<0.05$.

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DISCLOSURES

None.

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