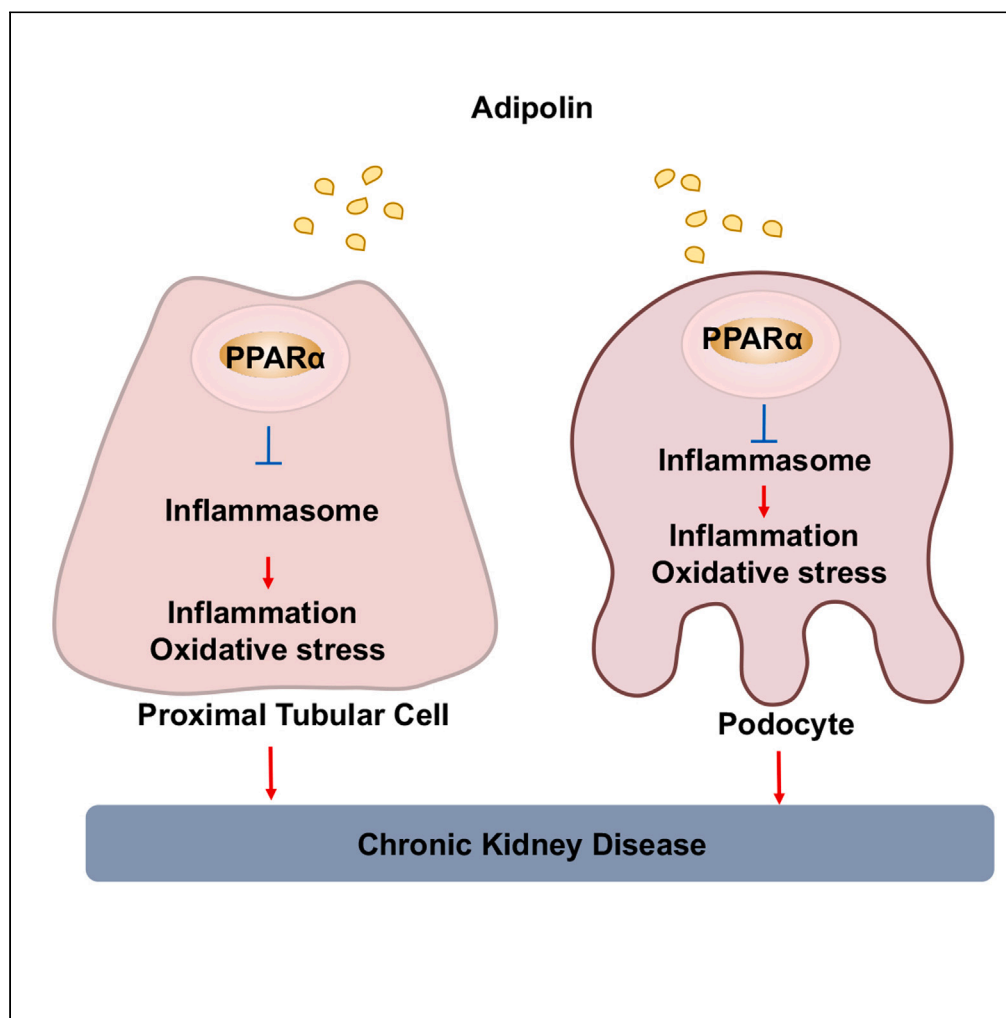


Article

Adipolin protects against renal injury via PPAR α -dependent reduction of inflammasome activation

Lixin Fang, Koji Ohashi, Satoko Hayakawa, ..., Ichihiro Shimomura, Toyoaki Murohara, Noriyuki Ouchi

ohashik@med.nagoya-u.ac.jp (K.O.)
nouchi@med.nagoya-u.ac.jp (N.O.)

Highlights

Adipolin deficiency enhances renal injury through activation of renal inflammasome

Adipolin prevents renal damage through PPAR α -dependent inflammasome inhibition

Adipolin reduces inflammasome activity in tubular cells via PPAR α /HMGC2 pathway

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Article

Adipolin protects against renal injury via PPAR α -dependent reduction of inflammasome activation

Lixin Fang,¹ Koji Ohashi,^{2,4,*} Satoko Hayakawa,¹ Hayato Ogawa,¹ Naoya Otaka,¹ Hiroshi Kawanishi,¹ Tomonobu Takikawa,¹ Yuta Ozaki,¹ Kunihiro Takahara,¹ Minako Tatsumi,² Mikito Takefuji,¹ Yuuki Shimizu,¹ Yasuko K. Bando,¹ Yuya Fujishima,³ Norikazu Maeda,³ Ichihiro Shimomura,³ Toyooki Murohara,¹ and Noriyuki Ouchi^{2,*}

SUMMARY

Although chronic kidney disease (CKD) is a major health problem worldwide, its underlying mechanism is incompletely understood. We previously identified adipolin as an adipokine which provides benefits for cardiometabolic diseases. Here, we investigated the role of adipolin in the development of CKD. Adipolin-deficiency exacerbated urinary albumin excretion, tubulointerstitial fibrosis and oxidative stress of remnant kidneys in mice after subtotal nephrectomy through inflammasome activation. Adipolin positively regulated the production of ketone body, β -hydroxybutyrate (BHB) and expression of a catalytic enzyme producing BHB, HMGCS2 in the remnant kidney. Treatment of proximal tubular cells with adipolin attenuated inflammasome activation through the PPAR α /HMGCS2-dependent pathway. Furthermore, systemic administration of adipolin to wild-type mice with subtotal nephrectomy ameliorated renal injury, and these protective effects of adipolin were diminished in PPAR α -deficient mice. Thus, adipolin protects against renal injury by reducing renal inflammasome activation through its ability to induce HMGCS2-dependent ketone body production via PPAR α activation.

INTRODUCTION

Chronic kidney disease (CKD) has become a major public health issue in recent years, with the number of cases worldwide increasing to 700 million in 2017.^{1,2} CKD contributes to an increased risk of cardiovascular mortalities. Obesity is an important risk factor for CKD development and participates in the pathogenesis of CKD, at least in part by increasing the risk of type 2 diabetes, atherosclerosis, and hypertension.³ Furthermore, obesity appears to promote CKD progression through its direct effects on the kidneys.^{3,4} However, the precise mechanism by which obesity causes CKD development has not yet been fully elucidated.

Adipose-derived hormonal factors, also known as adipokines, participate in the pathogenesis of various obesity disorders.^{5–7} Tumor necrosis factor (TNF) α and interleukin (IL) δ are pro-inflammatory adipokines, and their circulating levels are positively correlated with CKD prevalence.⁸ Leptin is upregulated by obesity and promotes glomerulosclerosis and renal fibrosis with pro-inflammatory properties.^{9,10} In contrast, adiponectin is an anti-inflammatory adipokine with reno-protective functions.^{11,12} Therefore, obesity may cause an imbalance of pro- and anti-inflammatory adipokines, leading to the development of CKD.

We previously identified adipolin, also referred to as C1q/Tnf-related protein 12, as an insulin-sensitizing adipokine downregulated by obesity.¹³ Adipolin improves insulin resistance in obese mice by attenuating adipose tissue inflammation. We also found that adipolin improves the pathological remodeling of vascular walls by reducing smooth muscle cell proliferation and macrophage inflammatory responses.¹⁴ Recently, we found that adipolin reduces adverse myocardial remodeling in response to myocardial infarction by suppressing inflammation.¹⁵ Thus, it is conceivable that adipolin acts as an anti-inflammatory adipokine that exerts beneficial actions on obesity-related metabolic and cardiovascular diseases. However, little is

¹Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan

²Department of Molecular Medicine and Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan

³Department of Metabolic Medicine, Osaka University Graduate School of Medicine, Osaka, Japan

⁴Lead contact

*Correspondence: ohashik@med.nagoya-u.ac.jp (K.O.), nouchi@med.nagoya-u.ac.jp (N.O.)

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Table 1. Characteristics of WT and APL-KO mice at 8 weeks after subtotal nephrectomy

	Sham		Nephrectomy	
	WT	APL-KO	WT	APL-KO
Body weight (g)	26.0 ± 0.4	25.3 ± 0.6	24.1 ± 0.4	24.2 ± 0.3
SBP (mmHg)	100.5 ± 3.4	101.4 ± 2.4	103.0 ± 2.1	101.1 ± 4.3
Heart rate (bpm)	624.7 ± 17.6	619.5 ± 5.4	627.8 ± 22.1	629.0 ± 15.9

Data are presented as mean ± S.E.M.

WT; wild-type mice, APL-KO; adipolin-knockout mice, SBP; systolic blood pressure, bpm: beat per minute.

known about the role of adipolin in renal diseases. Here, we investigated whether adipolin could modulate CKD development using a mouse subtotal nephrectomy model.

RESULTS

Disrupt of adipolin exacerbates renal damage following subtotal nephrectomy

To examine the role of adipolin in renal injury, adipolin-knockout (APL-KO) and wild-type (WT) mice were subjected to 5/6 nephrectomy. Eight weeks after subtotal nephrectomy or sham operation, APL-KO and WT mice were sacrificed for analyses after the collection of urine and blood samples. No differences were observed in body weight, systolic blood pressure, or heart rate between WT and APL-KO mice after subtotal nephrectomy or sham operation (Table 1). Subtotal renal ablation significantly increased urinary albumin excretion and circulating levels of urea nitrogen (UN) and creatinine (Cr) in WT mice (Figure 1A). Urinary albumin excretion and plasma UN levels were further increased in APL-KO mice after subtotal nephrectomy compared to those in WT mice (Figure 1A). In contrast, plasma Cr levels after the renal injury did not significantly differ between APL-KO and WT mice.

To evaluate interstitial fibrosis after renal injury, the kidneys of WT and APL-KO mice after subtotal nephrectomy or sham operation were stained with Masson's trichrome. APL-KO mice exhibited an increased fibrotic area in injured kidneys after subtotal renal ablation compared to WT mice (Figure 1B). APL-KO mice also showed increased expression levels of fibrosis markers, including collagen I, collagen III, and transforming growth factor (TGF) β 1, in the remnant kidney after partial nephrectomy compared to WT mice (Figure S1A).

To assess glomerular hypertrophy, the kidneys of WT and APL-KO mice after subtotal nephrectomy or sham operation were stained with Hematoxylin and Eosin (H-E). APL-KO mice exhibited an increased glomerular cross-sectional area after subtotal nephrectomy compared with WT mice (Figure S1B).

To evaluate renal tubular cell apoptosis, the kidneys of WT and APL-KO mice after subtotal nephrectomy or sham operation were stained with a terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL). APL-KO mice exhibited increased numbers of TUNEL-positive apoptotic cells in the remnant kidneys after subtotal nephrectomy compared with WT mice (Figure S1C). In contrast, no significant differences were observed in renal fibrosis area, glomerular cross-sectional area and renal apoptosis after sham operation between WT and APL-KO mice.

Inflammation, inflammasome activation, and oxidative stress contribute to CKD progression.^{16–19} Thus, to investigate the participation of inflammation, inflammasome activation, and oxidative stress in exacerbated renal injury in APL-KO mice, mRNA levels of inflammatory mediators, inflammasome-associated factors, and components of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the kidneys of WT and APL-KO mice were measured using quantitative real-time PCR. APL-KO mice exhibited higher mRNA levels of pro-inflammatory mediators, including TNF α , IL6, monocyte chemoattractant protein (MCP) 1 and F4/80 in the injured kidneys compared to WT mice (Figure 1C). In contrast, no differences were observed in mRNA levels of toll-like receptor (TLR) 2 and TLR4 in the injured kidneys between WT and APL-KO mice (Figure S1D). APL-KO mice also exhibited increased mRNA levels of inflammasome-related genes, including the nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing (NLRP) 3, caspase 1, IL1 β , and IL18 in the remnant kidneys after subtotal nephrectomy compared with WT mice (Figure 1D). Furthermore, APL-KO mice showed increased protein levels of NLRP3, caspase 1, and IL1 β in the remnant kidneys after subtotal nephrectomy compared to WT

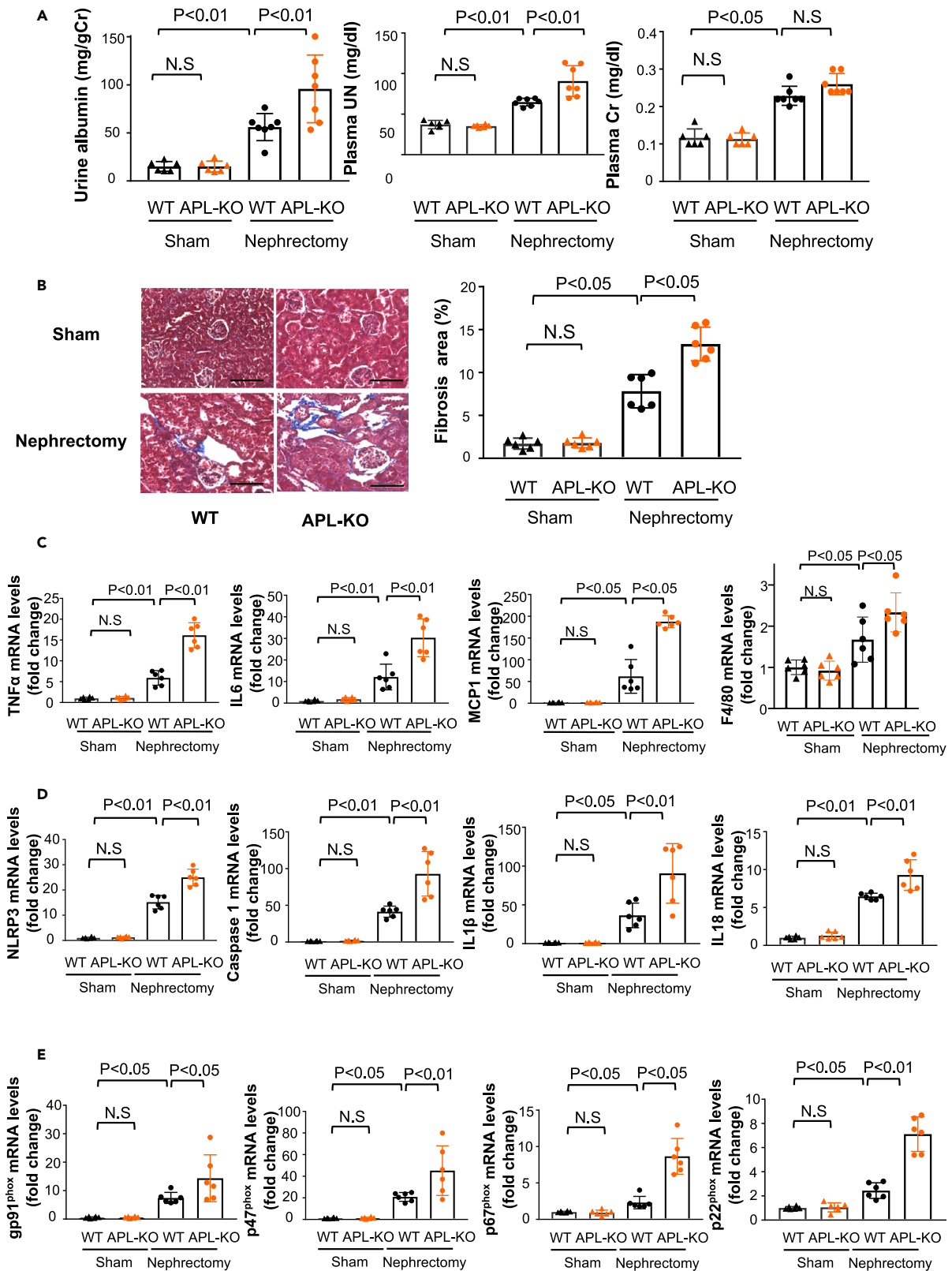


Figure 1. Adipolin-deficiency leads to exacerbation of albuminuria and tubulointerstitial fibrosis after renal ablation

(A) Urine and plasma parameters of renal function in wild-type (WT) and adipolin knockout (APL-KO) mice after subtotal nephrectomy or sham operation. Left panel shows urinary albumin excretion normalized to urinary Cr. Center and right panels show plasma concentration of UN and Cr. Sham WT group; N = 6, sham APL-KO group; N = 6, nephrectomy WT group; N = 7, nephrectomy APL-KO group; N = 7.

(B) Histological evaluation of interstitial fibrosis. Left panels show representative photos of the kidneys from WT and APL-KO mice after subtotal nephrectomy or sham operation as determined by Masson Trichrome staining. Right panel shows quantitative analysis of fibrosis area as measured by ImageJ. N = 6 in each group. Scale bars show 100 μ m.

(C) mRNA levels of tumor necrosis factor (TNF) α , interleukin (IL) 6, monocyte chemoattractant protein (MCP) 1 and F4/80 in the kidney in WT and APL-KO mice after subtotal nephrectomy or sham operation. N = 6 in each group.

(D) mRNA levels of inflammasome-related genes (NLRP3, Caspase 1, IL1 β and IL18) in the kidney in WT and APL-KO mice after subtotal nephrectomy or sham operation. N = 6 in each group.

(E) mRNA levels of oxidative stress markers (gp91^{phox}, p47^{phox}, p67^{phox} and p22^{phox}) in the kidney in WT and APL-KO mice after subtotal nephrectomy or sham operation. N = 6 in each group. One-way ANOVA with Tukey's multiple comparisons test ((A) (urine albumin and plasma UN), (C) (TNF α , IL6 and F4/80), (D) (NLRP3, Caspase 1 and IL18) and Steel-Dwass test ((A) (plasma Cr), (B), (C) (MCP1), (D) (IL1 β) (E) (p67^{phox})) were used to produce the p values. N.S, not significant.

mice (Figure S2). APL-KO mice also showed higher expression levels of gp91^{phox}, p47^{phox}, p67^{phox}, and p22^{phox}, which are components of NADPH oxidase, in the remnant kidneys after subtotal nephrectomy than WT mice (Figure 1E). In contrast, there were no significant differences in the expression levels of pro-inflammatory inflammasome-associated genes and components of NADPH oxidase in the kidneys after sham operation between WT and APL-KO mice (Figure 1E). Therefore, these data indicate that adipolin deficiency enhances renal damage following subtotal nephrectomy, with accompanying increases in inflammation, inflammasome activation, and oxidative stress in the kidney.

Systemic administration of adipolin ameliorates renal injury after subtotal nephrectomy

To assess the therapeutic effects of adipolin on renal injury, an adenoviral vector expressing adipolin (Ad-APL) or Ad- β -gal as control was intravenously injected into WT mice 4 weeks after subtotal nephrectomy. Ad-APL-treated WT mice showed a 2.2 ± 0.3 -fold increase in plasma adipolin levels 7 days after Ad-APL injection. Ad-APL administration significantly reduced urinary albumin excretion and plasma UN levels in WT mice 4 weeks after Ad-APL injection (i.e., 8 weeks after renal ablation) (Figure 2A). In contrast, Ad-APL treatment did not affect the plasma Cr levels in WT mice after subtotal nephrectomy. Ad-APL treatment reduced the fibrotic area in the remnant kidney of WT mice after subtotal nephrectomy compared with Ad- β -gal treatment (Figure 2B). Similarly, Ad-APL treatment significantly reduced the mRNA levels of fibrosis-related genes, including collagen I, collagen III, and TGF β 1 in the remnant kidneys of WT mice (Figure S3A). Ad-APL treatment also significantly reduced glomerular cross-sectional area in the injured kidneys in WT mice compared with Ad- β -gal treatment (Figure S3B). Moreover, Ad-APL treatment significantly reduced the numbers of TUNEL-positive apoptotic cells in the remnant kidneys of WT mice (Figure S3C). In addition, Ad-APL-treated WT mice showed reduced expression levels of TNF α , IL6, MCP1, F4/80, inflammasome-associated factors, including NLRP3, caspase 1, IL1 β , and IL18, and oxidative stress markers, including gp91^{phox}, p47^{phox}, p67^{phox}, and p22^{phox}, in the injured kidneys compared with Ad- β -gal-treated WT mice (Figures 2C and 2D).

Inhibition of inflammasomes attenuates exacerbated renal injury in APL-KO mice after subtotal nephrectomy

To investigate the contribution of inflammasome activation to enhanced renal damage in APL-KO mice, continuous administration of MCC950, an inhibitor of the NLRP3 inflammasome, was performed for 6 weeks (from 2 weeks to 8 weeks following subtotal nephrectomy). Compared with WT mice, APL-KO mice showed increased urinary albumin excretion and plasma UN levels, which were diminished by treatment with MCC950 (Figure 3A). In contrast, the treatment of WT mice with MCC950 did not significantly affect urinary albumin excretion or plasma UN levels. In addition, treatment of APL-KO mice with MCC950 significantly reduced tubulointerstitial fibrosis, glomerular cross-sectional area and the numbers of TUNEL-positive apoptotic cells in the remnant kidneys after subtotal nephrectomy (Figures 3B, S4A, and S4B). Treatment of APL-KO mice with MCC950 significantly attenuated the expression of inflammasome-associated genes and oxidative stress markers in injured kidneys (Figures 3C and 3D). These data indicate that adipolin deficiency contributes to enhanced inflammasome activation in response to renal ablation, thereby exacerbating the renal injury.

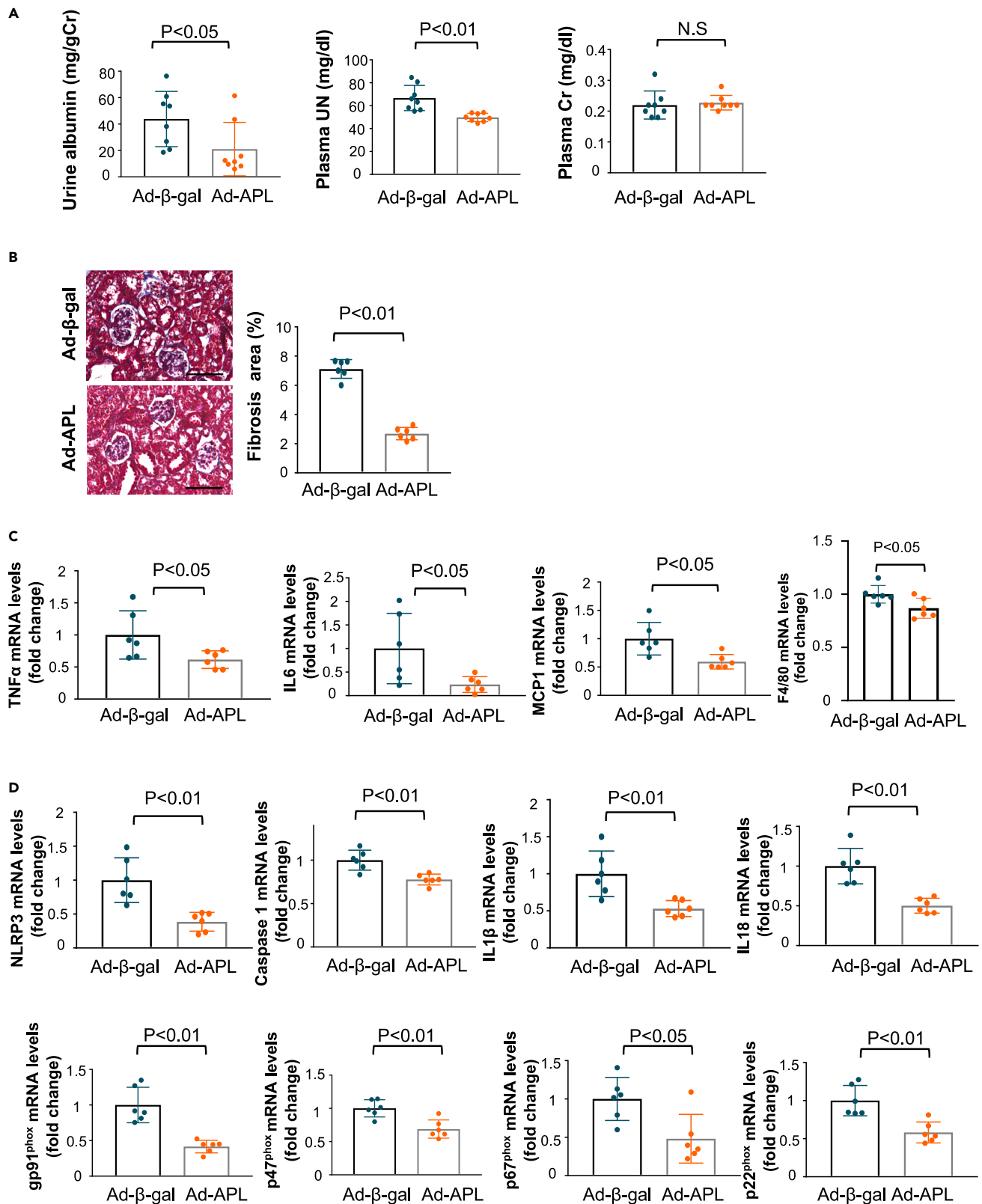


Figure 2. Systemic administration of adipolin ameliorates renal injury in WT mice after subtotal nephrectomy

(A) Urine and plasma parameters of renal function in WT mice treated with Ad- β -gal or Ad-APL at 8 weeks after subtotal nephrectomy. Left panel shows quantitative analysis of urinary albumin excretion normalized to urinary Cr. Center and right panels show plasma concentration of UN and Cr. N = 8 in each group.
 (B) Evaluation of interstitial fibrosis in the injured kidney. Left panels show representative photos of the remnant kidney from Ad- β -gal-treated or Ad-APL-treated WT mice as determined by Masson Trichrome staining. Right panel shows quantitative analysis of fibrosis area as measured by ImageJ. N = 6 in each group. Scale bars show 100 μ m.
 (C) mRNA levels of pro-inflammatory mediators (TNF α , IL6, MCP1 and F4/80) in WT mice treated with Ad- β -gal or Ad-APL.
 (D) mRNA levels of inflammasome-related genes (NLRP3, Caspase 1, IL1 β and IL18) and oxidative stress markers (gp91^{phox}, p47^{phox}, p67^{phox} and p22^{phox}) in WT mice treated with Ad- β -gal or Ad-APL. N = 6 in each group. Student's t-test ((A) (plasma UN), (B, C and D)) and Wilcoxon test ((A) (urine albumin and plasma Cr)) were used to produce the p values. N.S, not significant.

Adipolin suppresses activation of the inflammasome and oxidative stress in renal tubular cells and podocytes

To dissect the precise mechanism of adipolin-mediated attenuation of inflammasome activation, human renal proximal tubular epithelial cell line, HK-2 cells were pretreated with recombinant adipolin protein or vehicle, followed by stimulation with angiotensin (Ang) II. Pretreatment of HK-2 cells with adipolin significantly decreased Ang II-stimulated mRNA expression of inflammasome-related genes, including NLRP3, Caspase 1, IL1 β , and IL18 (Figure 4A). Consistently, pretreatment of HK-2 cells with adipolin significantly decreased the protein levels of NLRP3, Caspase 1, and IL1 β after stimulation with Ang II (Figure S5). In addition, pretreatment of HK-2 cells with adipolin attenuated Ang II-induced expression of oxidative stress markers, including gp91^{phox}, p47^{phox}, and p22^{phox} (Figure 4B).

To investigate whether adipolin modulates inflammasome activation and oxidative stress in podocytes, cultured podocytes, MPC-5 cells were pretreated with adipolin protein or vehicle, followed by stimulation with Ang II. Pretreatment of MPC-5 cells with adipolin significantly decreased Ang II-stimulated mRNA expression of inflammasome-related genes, including NLRP3, Caspase 1 and IL18, and oxidative stress markers, including gp91^{phox} and p47^{phox} (Figures S6A and S6B). Thus, it is conceivable that adipolin reduces inflammasome activation in renal tubular cells and podocytes, resulting in renal protection.

Adipolin increased the production of ketone bodies and β -hydroxybutyrate in the remnant kidney after subtotal nephrectomy

Recent reports have shown that ketone bodies exert beneficial effects on kidney disease.^{20,21} Thus, we assessed the levels of ketone body β -hydroxybutyrate (BHB) in the remnant kidney after subtotal nephrectomy. APL-KO mice showed reduced levels of renal BHB after subtotal nephrectomy compared with WT mice (Figure 5A). Systemic delivery of Ad-APL increased the renal BHB levels in WT mice in response to subtotal nephrectomy (Figure 5B). We also evaluated the expression of 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS) 2, a key enzyme producing BHB, in the remnant kidney. APL-KO mice showed reduced expression levels of HMGCS2 in injured kidneys following subtotal nephrectomy compared to WT mice (Figure 5C). Systemic administration of Ad-APL to WT mice increased HMGCS2 expression in the remnant kidney after subtotal nephrectomy (Figure 5D). Similarly, treatment with adipolin protein increased mRNA levels of HMGCS2 in HK-2 cells (Figure 5E).

To examine whether HMGCS2 was involved in the inhibitory effect of adipolin on inflammasome activation *in vitro*, HK-2 cells were transfected with siRNA targeting HMGCS2 or unrelated siRNA. Transduction of HK-2 cells with siRNA against HMGCS2 decreased mRNA levels of HMGCS2 by 80.2 \pm 2.0% (Figure S7). Ablation of HMGCS2 blocked the inhibitory effect of adipolin on Ang II-induced expression of NLRP3, Caspase 1, and IL1 β in HK-2 cells (Figure 5F). Thus, it is likely that adipolin could positively regulate ketone body production in the injured kidney through the induction of HMGCS2 expression, thereby contributing to the suppression of inflammasome activation.

Adipolin attenuates inflammasome activation in renal proximal tubular cells through the PPAR α /HMGCS2-dependent pathway

Because endogenous expression of HMGCS2 is induced by peroxisome proliferator-activated receptor (PPAR) α ,²² we assessed the effect of adipolin on PPAR α binding to peroxisome proliferator-activated receptor response element (PPRE) in HK-2 cells. Treatment of HK-2 cells with adipolin protein increased PPAR α binding to PPRE (Figure 6A). Similarly, adipolin treatment significantly increased the PPRE luciferase activity in HK-2 cells (Figure 6B). Adipolin treatment also increased the mRNA levels of lipoprotein lipase

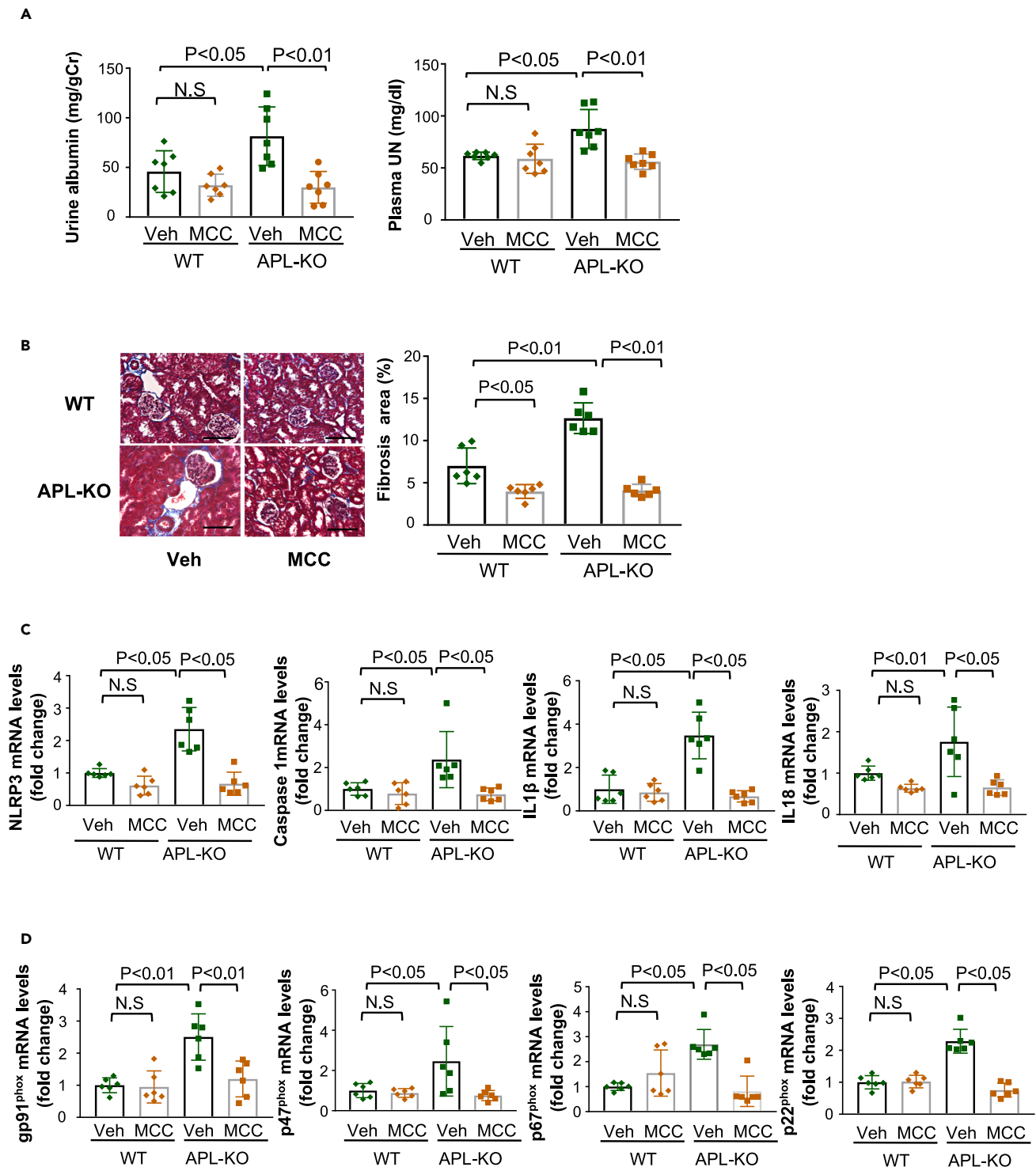


Figure 3. Inhibition of inflammasome activation ameliorates the exacerbated renal damage of APL-KO mice after subtotal nephrectomy

(A) Urine and plasma parameters of renal function in WT and APL-KO mice treated with vehicle (Veh) or MCC950 (MCC) at 8 weeks after subtotal nephrectomy. Left panel shows urinary albumin excretion normalized to urinary Cr. Right panel shows plasma concentration of UN. N = 7 in each group. (B) Evaluation of interstitial fibrosis in the injured kidneys. Left panels show representative photos of the remnant kidney from WT and APL-KO mice treated with vehicle (Veh) or MCC950 (MCC) as stained with Masson Trichrome. Right panel shows quantitative analysis of fibrosis area as measured by ImageJ. N = 6 in each group. Scale bars show 100 μ m.

Figure 3. Continued

(C and D) mRNA levels of inflammasome-related genes (NLRP3, Caspase 1, IL1 β and IL18) (D) and oxidative stress markers (gp91^{phox}, p47^{phox}, p67^{phox} and p22^{phox}) (E) in the remnant kidney in WT and APL-KO mice treated with vehicle (Veh) or MCC950 (MCC). N = 6 in each group. One-way ANOVA with Tukey's multiple comparisons test (A, B, C (IL18) and D) and Steel-Dwass test (C (NLRP3, Caspase1, IL1 β)) were used to produce the p values. N.S, not significant.

(LPL), uncoupling protein (UCP), and acyl CoA oxidase (ACOX), which are downstream target genes of PPAR α in HK-2 cells and MPC-5 podocytes (Figures 6C and 58). In addition, APL-KO mice showed reduced mRNA levels of LPL, UCP2, and ACOX1 in the remnant kidney after subtotal nephrectomy compared with WT mice (Figure 6D). Furthermore, Ad-APL administration to WT mice significantly increased the mRNA levels of LPL, UCP2, and ACOX1 in the remnant kidney after subtotal nephrectomy compared to Ad- β -gal treatment (Figure 6E). These findings indicate that adipolin promotes PPAR α -dependent signaling pathways in the injured kidneys.

To investigate the contribution of PPAR α to the adipolin-mediated inhibition of inflammasome activation in response to Ang II, HK-2 cells were treated with the PPAR α antagonist GW6471. Pretreatment of HK-2 cells with GW6471 reversed the suppressive effects of adipolin on the Ang II-stimulated expression of NLRP3, caspase 1, and IL1 β (Figure 6F). We previously reported that adipolin suppresses the inflammatory response of macrophages through TGF β /Smad signaling.¹⁴ We also showed that adipolin reduced cardiomyocyte apoptosis after myocardial infarction through the PI3-kinase/Akt-dependent pathway.¹⁵ Thus, to examine the possible involvement of TGF β /Smad signaling or PI3-kinase/Akt signaling in the suppressive actions of adipolin on Ang II-stimulated inflammasome activation, HK-2 cells were pretreated with an inhibitor of TGF β /Smad (SB431542) or an inhibitor of PI3-kinase/Akt (LY294002). Pretreatment of HK-2 cells with SB431542 or LY294002 did not affect the inhibitory effects of adipolin on the Ang II-stimulated increase in the expression of NLRP3, Caspase 1, and IL1 β (Figure 6F). Transduction of HK-2 cells with siRNA against PPAR α reduced the mRNA levels of PPAR α by 84.1 \pm 2.8% compared with transduction with unrelated siRNA as a control (Figure S9A). Knockdown of PPAR α by siRNA targeting PPAR α abrogated the adipolin-stimulated expression of HMGCS2 in HK-2 cells (Figure 6G). Moreover, ablation of PPAR α reversed the suppressive effects of adipolin on the Ang II-stimulated expression of NLRP3 and IL1 β in HK-2 cells (Figure S9B). These findings indicate that adipolin reduces inflammasome activation in proximal tubular cells through induction of the PPAR α /HMGCS2-dependent pathway (Figure 6H).

To dissect the upstream molecule of adipolin-induced PPAR α activation, HK-2 cells were transduced with siRNA targeting AdipoR1 or AdipoR2, or unrelated siRNA. Transduction of HK-2 cells with siRNA against AdipoR1 and AdipoR2 reduced the mRNA levels of AdipoR1 and AdipoR2 by 82.6 \pm 1.1% and 78.9 \pm 1.8% compared with transduction with unrelated siRNA (Figure S10A). Ablation of AdipoR2, but not knockdown of AdipoR1, abrogated the adipolin-stimulated expression of LPL and HMGCS2 in HK-2 cells. (Figure S10B). These results indicate that adipolin stimulates PPAR α activation in proximal tubular cells through an AdipoR2-dependent pathway.

Adipolin ameliorates renal injury after subtotal nephrectomy in a PPAR α -dependent manner

Finally, to assess the contribution of PPAR α to the renal protective effects of adipolin *in vivo*, Ad-APL or Ad- β -gal as control was intravenously injected into WT or PPAR α -knockout (KO) mice 4 weeks after subtotal nephrectomy. Although Ad-APL treatment significantly reduced urinary albumin excretion and plasma UN levels in WT mice, it did not affect urinary albumin excretion or plasma UN levels in PPAR α -KO mice (Figure 7A). In contrast to WT mice, Ad-APL treatment had no significant effect on tubulointerstitial fibrosis after subtotal nephrectomy in PPAR α -KO mice (Figure 7B). Similarly, Ad-APL treatment did not affect the expression levels of pro-inflammatory mediators, including TNF α and IL6, and inflammasome-associated factors, including NLRP3 and IL18 in the remnant kidney of PPAR α -KO mice after subtotal nephrectomy (Figure 7C). Collectively, adipolin exerts a renoprotective function by inhibiting inflammasome activation via a PPAR α -dependent pathway.

DISCUSSION

The present study provides the first evidence that adipolin exerts renoprotective effects in a mouse model of subtotal nephrectomy. Disruption of adipolin led to the enhancement of urinary albumin secretion, tubulointerstitial fibrosis, and renal inflammasome activation in mice following subtotal nephrectomy. Inflammasome inhibition by MCC950 blocked the enhancement of damaged kidney fibrosis under conditions of adipolin deficiency. Systemic administration of adipolin to mice with subtotal nephrectomy improved

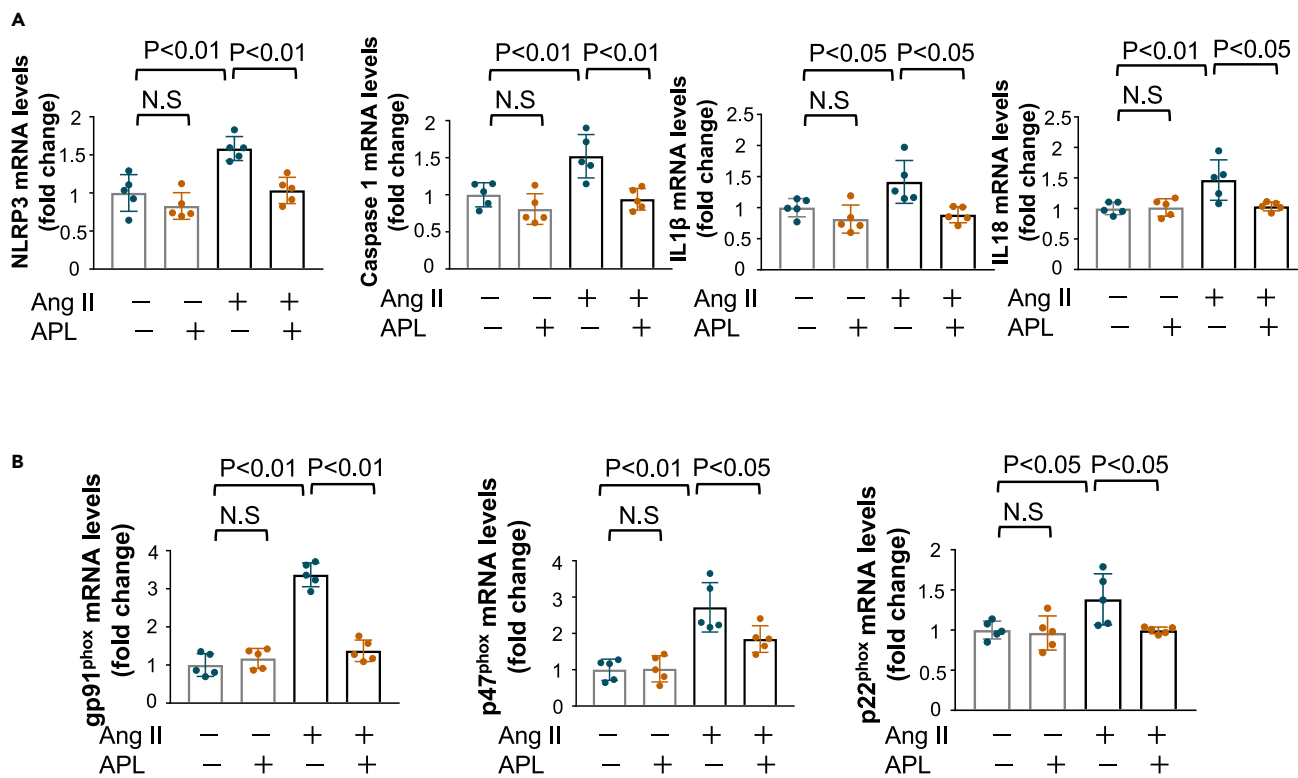


Figure 4. Adipolin attenuates activation of inflammasome and oxidative stress in renal proximal tubular cells

(A and B) Effect of adipolin (APL) on angiotensin (Ang) II-stimulated expression of inflammasome-related genes (NLRP3, Caspase 1, IL1 β and IL18) (A) and oxidative stress markers (gp91^{phox}, p47^{phox} and p22^{phox}) (B) in HK-2 cells. Human proximal tubular cell lines, HK-2 cells were pretreated with APL (300 ng/mL) or vehicle for 1 h followed by incubation in the presence or absence of Ang II (1 μ mol/L). N = 5 in each group. One-way ANOVA with Tukey's multiple comparisons test were used to produce the p values. N.S, not significant.

fibrosis and inflammasome activation in injured kidneys. Pretreatment of renal proximal tubular cells with adipolin attenuates inflammasome activation. These findings indicate that adipolin protects against renal dysfunction and injury by reducing inflammasome activation, suggesting that adipolin plays a crucial role in preventing CKD development.

NLRP3 inflammasome is involved in the pathogenesis of various non-microbial diseases, including CKD.^{23,24} NLRP3 deficient mice exhibit reduced tubular injury and fibrosis after unilateral urinary occlusion (UUO).¹⁸ Lack of apoptosis-associated speck-like protein containing CARD, an integral component of the inflammasome, results in attenuated renal injury and fibrosis after UUO.²⁵ Ang II has been reported to activate NLRP3-inflammasome in proximal tubular cells by increasing endoplasmic reticulum (ER) stress.²⁶ In addition, albumin dose-dependently increases expression of angiotensin converting enzyme (ACE) 1 in proximal tubular cells, leading to an increased production of Ang II.²⁷ Thus, it is likely that excessive exposure to albumin or Ang II could cause inflammasome activation in proximal tubular cells during CKD progression, thereby contributing to exacerbation of renal injury. Our present findings demonstrate that adipolin attenuates renal dysfunction and damage in a mouse model of subtotal nephrectomy by reducing inflammasome activation.

Ketone bodies, including BHB, are produced mainly in the liver and play an important role as an alternative energy source for the brain, heart, and skeletal muscle during nutrient starvation.^{28,29} Recently, sodium-glucose cotransporter 2 inhibitors were reported to protect against diabetic kidney disease by elevating BHB levels.²¹ Furthermore, it has been reported that BHB inhibits NLRP3 inflammasome activation in mouse models of NLRP3-mediated inflammation.³⁰ Our data indicated that adipolin positively regulates BHB production and HMGCS2 expression in injured kidneys. Our gene knockdown experiments also demonstrated that ablation of HMGCS2 canceled the adipolin-mediated reduction of Ang II-induced expression of inflammasome-related genes, including NLRP3, in renal proximal tubular cells. These results

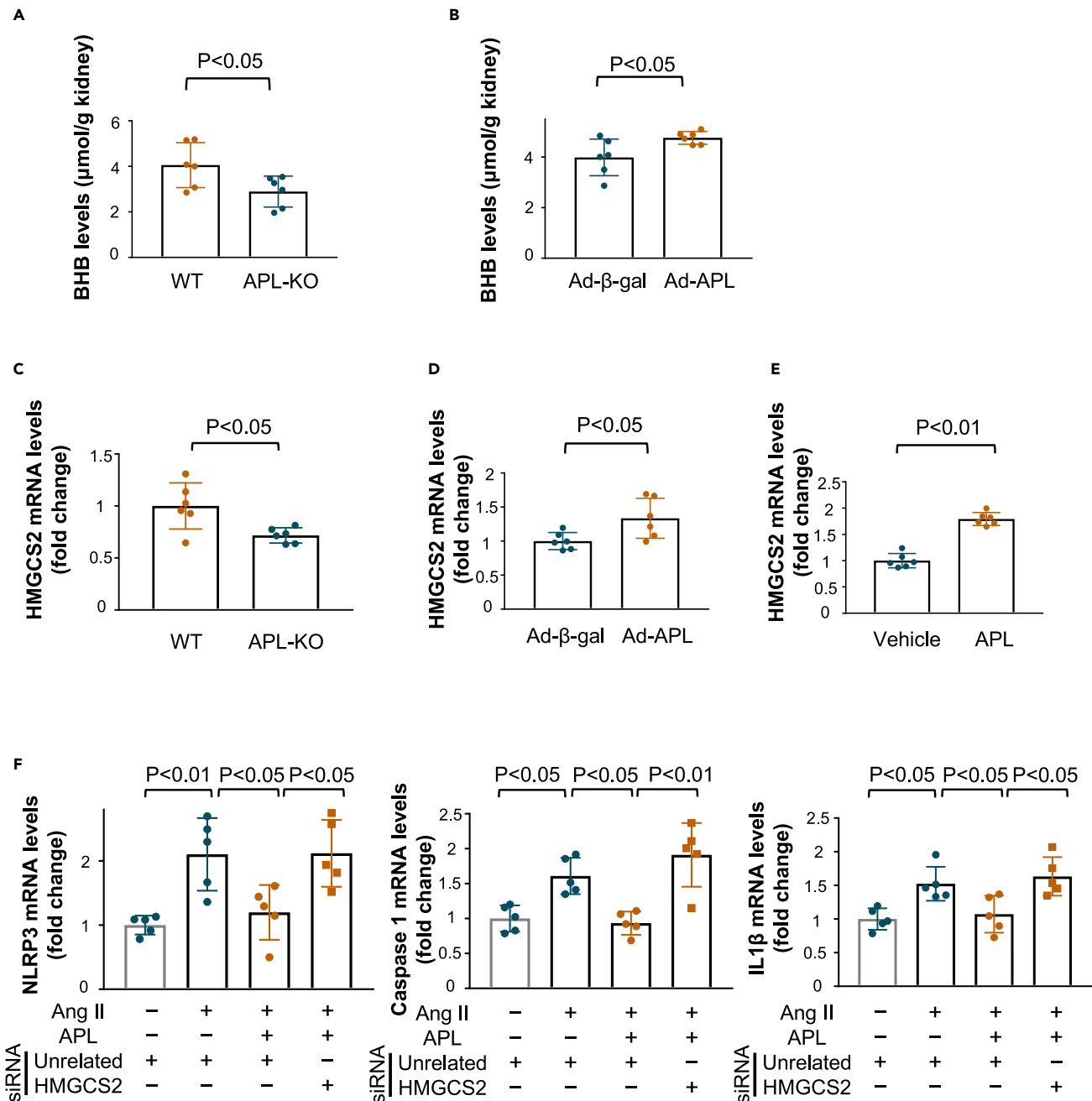


Figure 5. Adipolin positively regulates β -hydroxybutyrate production and HMGCS2 expression in the remnant kidney after subtotal nephrectomy

(A) β -hydroxybutyrate (BHB) levels in the remnant kidney from WT and APL-KO mice at 8 weeks after subtotal nephrectomy. N = 6 in each group.
 (B) BHB levels in the remnant kidney of WT mice treated with Ad- β -gal or Ad-APL at 8 weeks after subtotal nephrectomy. N = 6 in each group.
 (C) mRNA levels of HMGCS2, which is a key productive enzyme of BHB, in the remnant kidney of WT and APL-KO mice at 8 weeks after subtotal nephrectomy. N = 6 in each group.
 (D) mRNA levels of HMGCS2 in the remnant kidney of WT mice treated with Ad- β -gal or Ad-APL at 8 weeks after subtotal nephrectomy. N = 6 in each group.
 (E) mRNA levels of HMGCS2 in HK-2 cells treated with adipolin (APL) protein (300 ng/mL) or vehicle for 6 h. N = 6 in each group.
 (F) Contribution of HMGCS2 to the inhibitory effects of APL on Ang II-stimulated expression of inflammasome-related genes, including NLRP3, Caspase 1 and IL1 β in HK-2 cells. HK-2 cells were treated with siRNA targeting HMGCS2 (10 nmol/L) or control unrelated siRNA for 8 h. After 16 h of serum starvation, HK-2 cells were treated with APL protein (300 ng/mL) or vehicle for 1 h followed by stimulation with Ang II (1 $\mu\text{mol/L}$) or vehicle for 24 h. N = 5 in each group. Student's t-test (B, D and E), Wilcoxon test (A and C) and One-way ANOVA with Tukey's multiple comparisons test (F) were used to produce the p values.

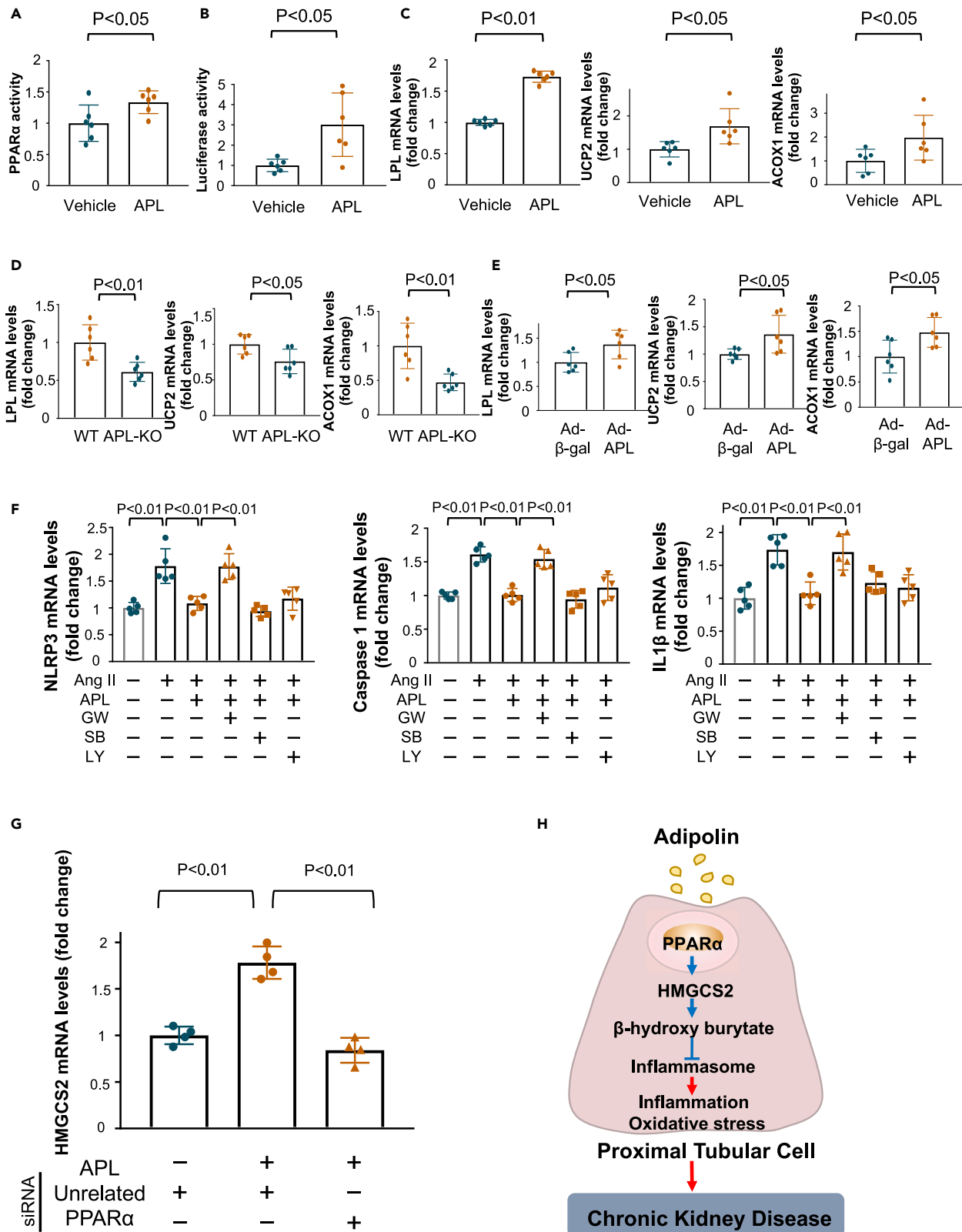


Figure 6. Adipolin reduces inflammasome activation in proximal tubular cells through the PPAR α /HMGCS2 pathway

- (A) PPAR α activity in HK-2 cells stimulated with adipolin (APL) protein (300 ng/mL) or vehicle for 6 h. N = 6 in each group.
 (B) PPRE luciferase activity in HK-2 cells stimulated with APL protein (300 ng/mL) or vehicle for 24 h. N = 6 in each group.
 (C) mRNA expression of lipoprotein lipase (LPL), uncoupling protein (UCP) 2 and acyl CoA oxidase (ACOX)-1, which are downstream molecules of PPAR α , in HK-2 cells after stimulation with APL protein (300 ng/mL) or vehicle for 6 h. N = 6 in each group.
 (D) mRNA levels of LPL, UCP2 and ACOX-1 in the remnant kidney of WT and APL-KO mice at 8 weeks after subtotal nephrectomy. N = 6 in each group.
 (E) mRNA levels of LPL, UCP2 and ACOX-1 in the remnant kidney of WT mice treated with Ad- β -gal or Ad-APL at 8 weeks after subtotal nephrectomy. N = 6 in each group.
 (F) Contribution of PPAR α , TGF β /Smad and PI3-kinase/Akt pathways to the suppressive effects of adipolin on angiotensin (Ang) II-induced expression of NLRP3, Caspase 1 and IL1 β in HK-2 cells. HK-2 cells were pretreated with GW6471 (GW; PPAR α antagonist, 50 μ mol/L), SB431542 (SB; TGF β /Smad inhibitor, 1 μ mol/L) or LY294002 (LY; PI3-kinase/Akt inhibitor, 20 μ mol/L) or vehicle for 30 min and treated with APL protein (300 ng/mL) or vehicle for 1 h followed by stimulation with Ang II (1 μ mol/L) or vehicle for 24 h. N = 5 in each group.
 (G) Contribution of PPAR α to the stimulatory effect of adipolin on HMGCS2 expression in HK-2 cells. HK-2 cells were pretreated with siRNA targeting PPAR α (10 nmol/L) or control unrelated siRNA for 24 h followed by treatment with APL protein (300 ng/mL) or vehicle for 6 h. N = 4 in each group.
 (H) Proposed scheme of the mechanism of renal protection by adipolin. Adipolin attenuates inflammation and oxidative stress by reducing inflammasome activation in proximal tubular cells through induction of PPAR α /HMGCS2/ β -hydroxybutyrate-dependent pathway, thereby leading to protection against chronic kidney disease. One-way ANOVA with Tukey's multiple comparisons test (F and G), Student's t-test (A–E) were used to produce the p values.

suggested that the ability of adipolin to protect against renal inflammasome activation *in vitro* and *in vivo* is dependent, at least in part, on its ability to increase ketone body production.

PPAR α is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors.³¹ PPAR α is abundantly expressed in the liver, skeletal muscle, heart, and kidney, and regulates lipid metabolism and the inflammatory response.^{32,33} Fenofibrate, a PPAR α agonist, reduces lipotoxicity-induced renal injury.³⁴ Fenofibrate also has a beneficial effect on fatty acid-induced apoptosis in renal tubular cells.³⁵ Transgenic mice expressing PPAR α in the proximal tubule show improved renal function during acute kidney injury.³⁶ These findings indicate that PPAR α activation contributes to protection against kidney disease. In the present study, we found that adipolin enhanced PPAR α activity and expression of PPAR α target genes in proximal tubular cells and that adipolin positively regulated the expression of PPAR α -dependent genes in the injured kidney of mice. Thus, it is likely that adipolin has a PPAR α ligand activity. HMGCS2 has a peroxisome proliferator response element in the promoter region and is transcriptionally regulated by PPAR α .²² In agreement with this finding, our experiments using siRNA showed that ablation of PPAR α blocked adipolin-induced expression of HMGCS2 in renal proximal tubular cells. Our *in vitro* experiments also showed that the suppressive effects of adipolin on Ang II-stimulated inflammasome activation in renal proximal tubular cells were abrogated by ablation of PPAR α or HMGCS2. Importantly, the protective effects of adipolin on injured kidneys *in vivo* were diminished in the background of PPAR α deficiency. Taken together, these data suggest that adipolin can attenuate renal inflammasome activation in response to subtotal renal ablation, at least in part, through its ability to increase HMGCS2 expression via PPAR α activation, thereby leading to renal protection (Figure 6H).

In conclusion, we demonstrated for the first time that adipolin, a novel adipokine, functions as a renoprotective factor that can mitigate renal inflammasome activity through a PPAR α /HMGCS2-dependent mechanism. We, and others, have shown that adipolin improves metabolic dysfunction, pathological remodeling of the vascular wall, and adverse cardiac remodeling after myocardial infarction.^{13–15,37} It has been reported that reduced levels of serum adipolin are associated with renal dysfunction in patients with type 2 diabetes.³⁸ Furthermore, patients with type 2 diabetes and hemodialysis exhibit significantly lower blood adipolin levels compared with patients with type 2 diabetes.³⁹ These clinical data indicate the inverse association between circulating adipolin levels and renal function. Thus, adipolin could represent a novel therapeutic target for the prevention or treatment of kidney and cardiometabolic diseases.

Limitations of the study

In the present study, we used mice subjected to subtotal (5/6) nephrectomy as CKD models. Although the mouse 5/6 nephrectomy model is widely used, this model shows mild renal dysfunction compared with other models of CKD such as lupus nephritis and diabetic kidney disease. In addition, we used 5/6 nephrectomized WT mice fed normal diets. It would be better to employ obese mice to clarify the role of adipolin in obesity-associated CKD. Thus, future studies will be needed to dissect the role of adipolin in severe renal dysfunction using different CKD models.

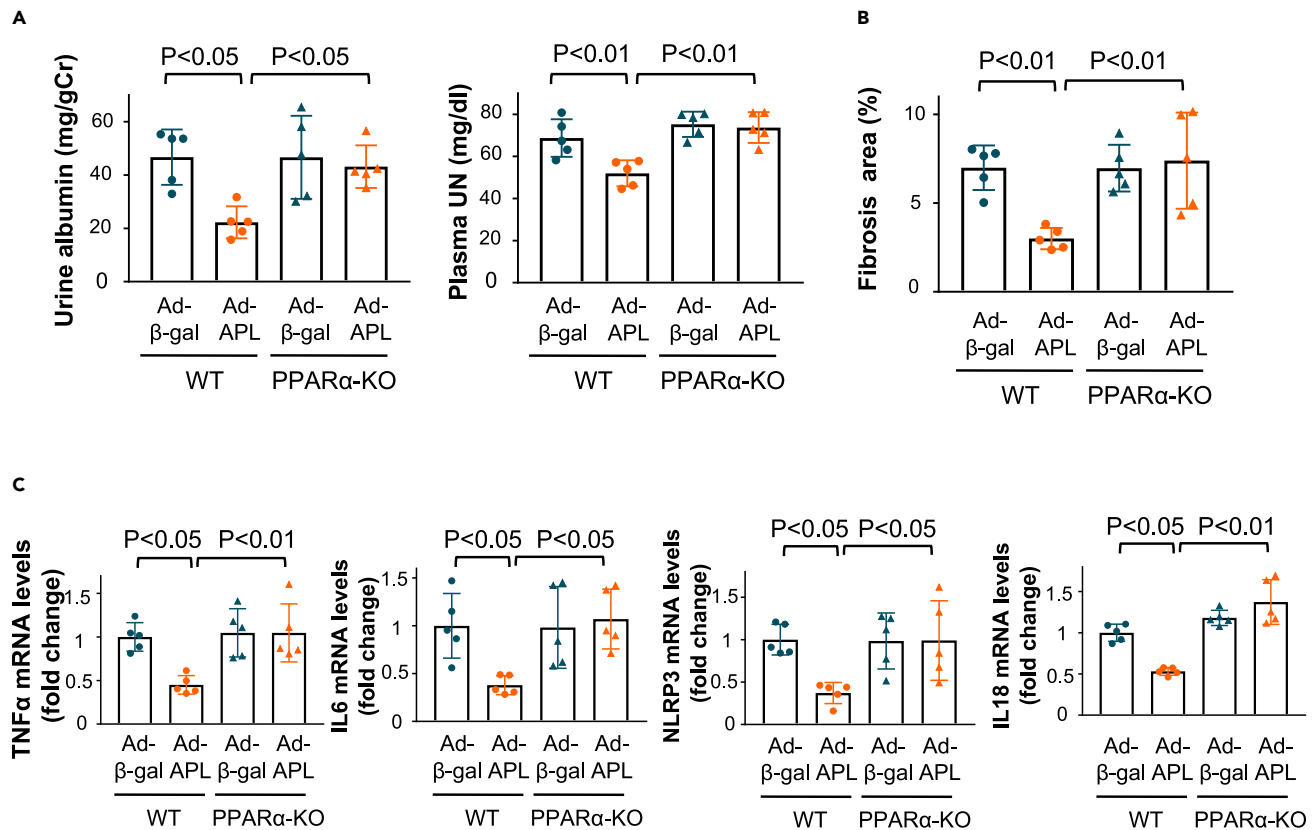


Figure 7. Systemic administration of adipolin ameliorates renal injury after subtotal nephrectomy through the PPAR α dependent mechanism *in vivo*

(A) Urine and plasma parameters of renal function in WT and PPAR α knockout (KO) mice treated with Ad- β -gal or Ad-APL at 8 weeks after subtotal nephrectomy. Left panel shows quantitative analysis of urinary albumin excretion normalized to urinary Cr. Right panel shows plasma concentration of UN. N = 5 in each group.

(B) Evaluation of interstitial fibrosis as assessed by Masson Trichrome staining. Quantitative analysis of fibrosis area in the injured kidneys from WT and PPAR α -KO mice treated with Ad- β -gal or Ad-APL as measured by ImageJ. N = 5 in each group.

(C) mRNA levels of pro-inflammatory mediators (TNF α and IL6) and inflammasome-related genes (NLRP3 and IL18) in the injured kidneys in WT mice and PPAR α -KO mice treated with Ad- β -gal or Ad-APL. N = 5 in each group. One-way ANOVA with Tukey's multiple comparisons test were used to produce the p values.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2023.106591>.

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AUTHOR CONTRIBUTIONS

L.F. designed the research study, conducted the experiments, acquired the data, analyzed the data and wrote the manuscript. K.O. designed the research study, conducted the experiments, acquired the data, analyzed the data, provided expertise related to the experiments and wrote the manuscript. S.H., H.O., N. Otaka, H.K., T.T., Y.O., K.T., and M. Tatsumi conducted the experiments, acquired the data, analyzed the data. M. Takefuji, Y.S., Y.B.K., and T.M. designed the research study, provided expertise related to the experiments. Y.F., N.M., and I.S. provided PPAR α deficient mice and expertise related to the experiments. N. Ouchi designed the research study, analyzed the data, provided expertise related to the experiments and wrote the manuscript. All authors participated in interpreting the results and revising the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-NLRP3 antibody	Adipogen	AG-20B-0014
Anti-NLRP3 antibody	Novus	NBP2-12446SS
Anti-Caspase 1 antibody	Adipogen	AG-20B-0042
Anti-Caspase 1 antibody	Proteintech	22915-1-AP
Anti-IL1 β antibodies	R&D Systems	AF-401-SP
Bacterial and virus strains		
Adenovirus producing β -galactosidase	This paper	N/A
Adenovirus producing adipolin	This paper	N/A
Chemicals, peptides, and recombinant proteins		
MCC950	Adipogen	AG-CR1-3615
GW6471	Cayman	11697
SB431542	Cayman	13031
LY294002	Cell Signaling Technology	#9901
Recombinant angiotensin II protein	Sigma-Aldrich	A9525
Critical commercial assays		
Masson's trichrome assay kit	Sigma-Aldrich	HT15
β -hydroxybutyrate and a ketone Body assay kit	Cayman	700190
TUNEL Assay kit-HRP-DAB	Abcam	AB20638
Plasma UN and Cr measurement	SRL	commercial laboratory
Urine Cr measurement	SRL	commercial laboratory
murine albumin enzyme-linked immunosorbent assay kit	Exocell	1011
PPAR α transcription factor assay kit	Cayman	10006915
dual-luciferase reporter assay system	Promega	E1910
Experimental models: Cell lines		
Human HK-2 cell	ATCC	CRL2190
Mouse MPC-5 podocyte clonal cell	University of Bristol	N/A
Experimental models: Organisms/strains		
Mouse C57BL/6J	Jackson Laboratories	Strain No. 000664
Adipolin knockout mice	This paper	N/A
PPAR α knockout mice (B6; 129S4- <i>Pparα1Gonz/J</i>)	Jackson Laboratories	Strain No. 008154
Oligonucleotides		
siRNA targeting nontargeting pool	Horizon Discovery	D-001810-10
siRNA targeting HMGCS2 (ON-TARGETplus, SMARTpool)	Horizon Discovery	L-010179-00
siRNA targeting PPAR α (ON-TARGETplus, SMARTpool)	Horizon Discovery	L-003434-00
siRNA targeting AdipoR1 (ON-TARGETplus, SMARTpool)	Horizon Discovery	L-007800-01

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
siRNA targeting AdipoR2 (ON-TARGETplus, SMARTpool)	Horizon Discovery	L-007801-01
Software and algorithms		
image J analysis system	Schneider et al. ⁴⁰	Nat Methods 9, 671–675. 10.1038/nmeth.2089
JMP Pro 15 software	SAS	N/A

RESOURCE AVAILABILITY**Lead contact**

Further information and requests of resources and reagents should be directed to the lead contact, Koji Ohashi (ohashik@med.naogya-u.ac.jp).

Materials availability

Antibodies were commercial sources described in the STAR Methods [key resources table](#). All unique materials generated in this study will be available from the lead contact after completing a Material Transfer Agreement.

Data and code availability

- All data for evaluating the contributions in the paper are presented in the paper and/or the [supplemental information](#).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Animal and surgical procedure**

Adipolin knockout (APL-KO) mice were generated as previously described (Accession No. CDB1102K website: <http://www2.clst.riken.jp/arg/mutant%20mice%20list.html>).¹⁴ Homozygous APL-KO and wild-type (WT) mice with a C57BL/6J background at 8 weeks of age were used in this study. The genotyping primers for the adipolin (Fam 132a/CTRP12) WT allele were as follows: 5'-GCCTGAATCCCCCTAACT-3' (P1) and 5'-TCTGGTAGCCCTGAGAATCG-3' (P2). Primers for the adipolin (Fam 132 α /CTRP12) null allele were as follows: 5'-GGAAGTGCCCAATGAGTCC-3' (P3) and 5'-GTGGATGTGGAAATGTGTGC-3' (P4). PPAR α knockout (PPAR α -KO) mice were purchased from the Jackson Laboratory at Osaka University and were provided by Yuya Fujishima, Norikazu Maeda, and Ichihiro Shimomura at Osaka University.⁴¹ Male APL-KO and WT mice at the age of 8 weeks were assigned to two groups with or without subtotal renal ablation. Subtotal (5/6 of) nephrectomies were performed using surgical excision.^{11,42} Briefly, the upper and lower poles of the left kidney (two-thirds of the left kidney) were resected. After one week, the remaining right kidney was removed through a right paramedian incision after ligation of the right renal artery, vein, and ureter. Eight weeks after ablation, APL-KO and WT mice were sacrificed for analysis. In some experiments, Alzet mini-osmotic pumps (Model 2006, Durect Corp.) were used to deliver 5 mg/kg/day of MCC950 (Adipogen) two weeks after ablation for a period of 42 days. Pumps were implanted subcutaneously in the murine mid-scapular region through a small incision in the back of the neck, which was closed with clips. Before the surgical procedure, anesthesia (medetomidine, midazolam, and butorphanol at doses of 0.15, 2.0, and 2.5 mg/kg, respectively) was administered intraperitoneally. Adequacy of anesthesia was confirmed by the lack of a toe-pinch withdrawal response during the surgical procedure. In some experiments, Ad-APL, or Ad- β -gal (2×10^9 plaque-forming units/mouse) was intravenously injected into WT or PPAR α -KO mice 4 weeks after renal ablation. The study protocol was approved by the Institutional Animal Care and Use Committee of Nagoya University. All animal procedures were performed in accordance with the National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals).

Cell culture

HK-2 cells were purchased from AmericanType Culture Collection (Rockville, MD, USA). The cells were cultured in a medium consisting of DMEM/F12 (Gibco) supplemented with 10% FBS at intervals of 3–4 days to continuously passaged. HK-2 cells were cultured in DMEM/F12 medium for 16 h and cultured in the presence or absence of adipolin (300 ng/ml) for 1 h, followed by stimulation with Ang (1 μ mol/L) or vehicle for 24 h. Knockdown of PPAR α , HMGCS2, AdipoR1, or AdipoR2 was achieved by siRNA transduction at 10 nM with Lipofectamine RNAiMAX (Invitrogen) 24 h before experiments. Lipofectamine RNAiMAX and siRNAs were dissolved in Opti-MEM (Gibco). The ON-TARGETplus siRNA SMART pools targeting each gene were purchased from Horizon Discovery. Control cultures were transfected with an unrelated scrambled siRNA (ON-TARGET plus Control Non-Targeting Pool, Horizon Discovery).

Conditionally immortalized murine MPC-5 podocyte clonal cells (University of Bristol) were maintained in RPMI1640 medium containing 10% fetal bovine serum, 10 IU/ml recombinant murine interferon- γ (Wako) at 33°C for proliferating cells. To induce differentiation, MPC-5 cells were incubated at 5% CO₂ at 37°C without interferon- γ for 14 days.

METHOD DETAILS

Materials

A polyclonal antibody against mouse adipolin was generated, as previously described.⁴³ Anti-NLRP3 antibody was purchased from Adipogen and Novus. Anti-caspase 1 antibodies were purchased from Adipogen and Proteintech. Anti-IL1 β antibodies were purchased from R&D Systems. MCC950 was purchased from Adipogen. GW6471 and SB431542 were purchased from Cayman Chemicals. LY294002 was purchased from Cell Signaling Technology. Recombinant angiotensin II protein was purchased from Sigma-Aldrich. β -hydroxybutyrate and a ketone Body assay kit were purchased from Cayman. Adenoviral vectors expressing the genes encoding β -galactosidase (Ad- β -gal) and FLAG-tagged adipolin (Ad-APL) were constructed under the control of the CMV promoter.^{13,14}

Histological analyses

Tissue samples were fixed with 4% paraformaldehyde and embedded in paraffin. Serial tissue sections (5 μ m) of the kidneys were stained with Masson's trichrome (Sigma-Aldrich) and hematoxylin and eosin (H-E). Renal apoptosis was assessed by a terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) staining using TUNEL Assay kit-HRP-DAB (Abcam). The fibrotic area, glomerular size and apoptotic cell number were measured by using an image J analysis system.^{11,40}

Laboratory methods

Eight weeks after the operation, the mice were sacrificed, and blood and urine samples were collected for analysis. Plasma concentrations of urea nitrogen (UN) and creatinine (Cr) and urine concentrations of Cr were measured in a commercial laboratory (SRL). The urinary albumin concentration was measured using a murine albumin enzyme-linked immunosorbent assay kit (Exocell). Urinary albumin excretion was evaluated as albumin per gram of urinary Cr. The specific DNA-binding activity of PPAR α was evaluated using a PPAR α transcription factor assay kit (Cayman).

Western blot analysis

Tissue samples and cell samples were prepared in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1% NP-40, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma Chemical Co.). The protein concentration was determined by a BCA protein assay kit (Thermo Scientific). The equal amounts of proteins were separated by denaturing SDS-PAGE. Proteins were transferred onto PVDF membrane (GE Healthcare) and probed with the primary antibody followed by incubation with the HRP-conjugated secondary antibody. ECL prime system (GE Healthcare) were used for detection of the protein signal. The expression levels were determined by measurement of the corresponding band intensities by using image J software (National Institute of Health),⁴⁰ and the relative values were expressed relative to α -tubulin signal.

Luciferase assay

HK-2 cells in 24 well dishes were transfected with 500 ng of ptkLUC vector (Promega) containing a fragment of human PPRES along with 500 ng of pCMV6-AC-GFP (PS100010) expression vector (Origene) containing

human PPAR α in the presence of 50 ng of pRL-SV40 (Promega) using Lipofectamine 2000, as previously described.⁴⁴ After 24 h, cells were lysed in Passive Lysis Buffer (Promega), and the lysates were assayed for firefly and Renilla luciferase activities using a dual-luciferase reporter assay system (Promega). Promoter activity was determined as the ratio of firefly luciferase activity to Renilla luciferase activity.

Determination of mRNA levels

Gene expression levels were analyzed using quantitative real-time PCR. RNA from HK-2 cells and kidney tissue was extracted using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using ReverTra Ace (TOYOBO). Real-time PCR was performed with a Bio-Rad real-time PCR detection system using the THUNDERBIRD SYBR qPCR mix (TOYOBO) as a double-standard DNA-specific dye. The primers used are listed in [Tables S1](#) and [S2](#). The expression levels of the examined transcripts were compared with those of 36B4 and normalized to the mean value of the controls.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as mean \pm S.E.M. The differences in variables with normal distributions between the two groups were evaluated using an unpaired Student's t-test. Differences between three or more groups were evaluated using one-way analysis of variance (ANOVA) with post-hoc Tukey's test. Differences between groups for variables with non-normal distribution were analyzed using the Wilcoxon signed-rank test (for two groups) or the Steel-Dwass test (for three or more groups). Data distributions were evaluated using the Shapiro-Wilk test. $P < 0.05$ denoted the presence of a statistically significant difference. All statistical analyses were performed using the JMP Pro 15 software (SAS).