Exacerbation of Albuminuria and Renal Fibrosis in Subtotal Renal Ablation Model of Adiponectin-Knockout Mice

Koji Ohashi, Hirotsgu Iwatani, Shinji Kihara, Yasuhiko Nakagawa, Noriyuki Komura, Koichi Fujita, Norikazu Maeda, Makoto Nishida, Fumie Katsube, Ichiro Shimomura, Takahito Ito, Tohru Funahashi

Objective—Obesity is recognized increasingly as a major risk factor for kidney disease. We reported previously that plasma adiponectin levels were decreased in obesity, and that adiponectin had defensive properties against type 2 diabetes and hypertension. In this study, we investigated the role of adiponectin for kidney disease in a subtotal nephrectomized mouse model.

Methods and Results—Subtotal (5/6) nephrectomy was performed in adiponectin-knockout (APN-KO) and wild-type (WT) mice. The procedure resulted in significant accumulation of adiponectin in glomeruli and interstitium in the remnant kidney. Urinary albumin excretion, glomerular hypertrophy, and tubulointerstitial fibrosis were significantly worse in APN-KO mice compared with WT mice. Intraglomerular macrophage infiltration and mRNA levels of vascular cell adhesion molecule (VCAM)-1, MCP-1, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β1, collagen type I/III, and NADPH oxidase components were significantly increased in KO mice compared with WT mice. Treatment of APN-KO mice with adenovirus-mediated adiponectin resulted in amelioration of albuminuria, glomerular hypertrophy, and tubulointerstitial fibrosis and reduced the elevated levels of VCAM-1, MCP-1, TNF-α, TGF-β1, collagen type I/III, and NADPH oxidase components mRNAs to the same levels as those in WT mice.

Conclusions—Adiponectin accumulates to the injured kidney, and prevents glomerular and tubulointerstitial injury through modulating inflammation and oxidative stress. (Arterioscler Thromb Vasc Biol. 2007;27:1910-1917.)

Key Words: adiponectin ■ obesity ■ subtotal nephrectomy ■ inflammation ■ oxidative stress

Obesity is recognized increasingly as a major risk factor for kidney disease. It has been reported that body mass index (BMI) was associated significantly with increased risk for chronic kidney disease after adjusting for other confounders.1

The adipose tissue produces and secretes many bioactive substances, known as adipokines, which directly contribute to obesity-linked metabolic and vascular diseases. Adiponectin is an adipocyte-specific plasma protein that was identified in our laboratories in a human adipose tissue cDNA library.2 In a series of publications, we reported that physiological concentrations of human recombinant adiponectin suppressed the expression of endothelial adhesion molecules, vascular smooth muscle cell (VSMC) proliferation, macrophage-to-foam cell transformation, and tumor necrosis factor (TNF)-α production by macrophages in vitro.3–5 We have also shown that adiponectin selectively increased the expression of tissue inhibitor of metalloproteinases, which inhibits extracellular matrix degradation and protects the vascular wall from plaque disruption, in human monocye-derived macrophages through interleukin (IL)-10 induction, an anti-inflammatory cytokine.6 Recently, it has been reported that adiponectin exhibited cardioprotective effects after myocardial ischemia/reperfusion through the reduction of oxidative stress.7 In human studies, we also reported the presence of hypoadiponectinemia in patients with obesity, type 2 diabetes mellitus, and coronary artery disease.8–10 Interestingly, plasma adiponectin levels are an inverse predictor of cardiovascular outcomes in patients with end-stage renal disease11 even though plasma adiponectin levels show a negative correlation with GFR through unknown mechanisms.12

Experimental subtotal (5/6) renal ablation of mice and rats results in progressive glomerular hypertrophy, podocyte injury, and subsequent fibrosis and proteinuria through adaptive glomerular hyperfiltration,13,14 oxidative stress,7 and inflammation.15 We performed the renal mass reduction by surgical resection of the poles of the kidney rather than by renal artery ligation to prevent the development of severe hypertension, which would be a significant confounding factor in the assessment of the effects of microvascular injury on renal progression.16 In the present study, we investigated the role of adiponectin against glomerular and tubulointerstitial injury
using adiponectin-knockout (KO) mice burdened with subtotal renal ablation.

Methods

Animal and Animal Treatment
KO mice were generated as described previously and backcrossed to wild-type (WT) C57BL/6J.17 Both APN-KO and WT male mice (8- to 10-week-old) were assigned to 2 groups with or without subtotal renal ablation. Subtotal (5/6) nephrectomy was performed by the surgical excision method.18 All surgical procedures were carried out under anesthesia with intraperitoneal pentobarbital (30 mg/kg body wt; Sigma). The left kidney was exposed through a left paramedian incision and then decapsulated, leaving the adrenal gland intact. The upper and lower poles (two-thirds of the left kidney) were resected, and the remnant kidney was allowed to recover for 1 week. Then 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, KO and WT mice were euthanized for analysis. Tissues were fixed by perfusion of 10% buffered formalin via heart and subsequent immersion in 10% buffered formalin at 4°C for 4 hours. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

Histology and Immunohistochemistry
Four-μm paraffin or optimal cutting temperature (OCT) compound (Sakura) -embedded sections were analyzed immunohistochemically by use of rabbit polyclonal anti-mouse adiponectin antibody (Otsuka Pharmaceutical), goat monoclonal anti-mouse F4/80 antibody (Ce-darlane) and goat polyclonal anti-mouse nphrin antibody (Santa Cruz). After incubation with biotin-conjugated secondary antibody, as to adiponectin and nphrin antibody, the specimens were processed by use of the avidin-biotin-peroxidase complex kit (Vector Laboratories). Peroxidase activity was detected with a Liquid DAB Substrate Kit (Zymed Laboratories Inc). As to F4/80, the fluorescent-labeled secondary antibody, Alexa Fluor 488-conjugated guinea pig anti-goat antibody (Molecular Probes), was used. To analyze renal fibrosis, paraffin-embedded sections were stained with periodic acid-Schiff (PAS) method and Masson trichrome method. More than 20 consecutive sections in each mouse were examined, and the mean number of macrophages in the glomeruli was calculated. The number of cells was determined from light microscopic images (Provis AX 80 equipped with an HDTV system and a color-chilled 3 charged coupled device camera; Olympus) using an image analysis system (Macscope version 2.55; Mitani).

Blood Pressure Measurement
Systolic blood pressure (SBP) and heart rate (HR) were measured using the tail cuff technique with an automatic sphygmomanometer (BP98A; Softron) at the tail artery while the animals were restrained. Mice were trained to the tail cuff apparatus at least twice. Ten readings were taken for each measurement, and a mean value was assigned to each individual mouse.

Laboratory Methods
Blood samples were obtained from the retroorbital sinus from these mice before and 4, 6, and 8 weeks after ablation. Spontaneously voided urine was collected between 8 and 11 AM. The blood concentrations of urea nitrogen and creatinine were measured by using appropriate biochemical methods in a commercial laboratory (SRL). Creatinine clearance, in microliters of plasma and urine per minute was calculated by creatinine clearance CCr=(Cu/Cp)×V, where Cu is the concentration of creatinine in urine, Cp is the concentration of creatinine in plasma at the time of a 24-hour urine collection, and V is the urine flow rate in microliters per minute. Urinary albumin excretion was assayed with a murine albumin enzyme-linked immunosorbent assay kit (Exocell). To standardize urinary albumin excretion for GFR, albuminuria was expressed as milligrams of urinary albumin per gram of urinary creatinine. Adiponectin concentrations were determined with ACRP30 ELISA kits (Otsuka Pharmaceutical Co).

Gene Expression Analysis
Total RNA was extracted using RNA-STAT kit (TEL-TEST) according to the protocol supplied by the manufacturer, and 0.5 μg RNA was reverse-transcribed using the ThermoScript RT-PCR system (Invitrogen). Real-time PCR was performed on a LightCycler using the FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the protocol provided by the manufacturer.

We used the primers listed in supplemental materials (available online at http://atvb.ahajournals.org). All results were normalized to 36B4.

Preparation and Delivery of Adenoviral Adiponectin
Adenovirus producing the full-length adiponectin was constructed with Adenovirus Expression Vector Kit (TaKaRa) as described previously.17 Then, 5x1010 plaque-forming units of adenovirus-adiponectin (Ad-APN) or adenovirus β-galactosidase (Ad-β gal) was injected intravenously via the tail vein.

Statistical Methods
Data are presented as mean±SEM. Differences between groups were evaluated by the Student t test or analysis of variance (ANOVA) with Fisher PLSD test. A probability value less than 0.05 denoted the presence of a statistically significant difference. All calculations were performed by using a standard statistical package (StatView for Macintosh, version 5.0).

Results

Accumulation of Adiponectin in Glomeruli and Interstitium in Remnant Kidney
Immunohistochemical analysis showed abundant immunostaining for adiponectin in the glomeruli and interstitium of the remnant kidney of WT mice at 8 weeks after subtotal nephrectomy but not in the control glomeruli and interstitium (Figure 1a to 1d). However, real-time PCR showed no detectable level of adiponectin mRNA in the remnant kidney of WT mice (data not shown). These findings indicate accumulation of adiponectin in the glomeruli and interstitium in the injured kidney.

Severe Glomerular Hypertrophy and Tubulointerstitial Fibrosis in Subtotal-Nephrectomized Adiponectin-KO Mice
Subtotal nephrectomy resulted significant rise in urinary excretion of albumin in KO mice but not in WT mice (Table). On the other hand, subtotal nephrectomy did not significantly change levels of blood urea nitrogen, Cr concentrations, creatinine clearance, body weight, SBP, heart rate (HR), or number of glomeruli per section in KO compared with WT mice (Table). Importantly, blood pressure remained within the normal range during our study both in KO and WT mice.

Eight weeks after subtotal nephrectomy, kidney sections of WT mice showed mild glomerular hypertrophy, increased intraglomerular cells mostly in the mesangial area, and tubulointerstitial fibrosis (compare Figure 2a and 2b with 2e and 2f). These changes were more severe in adiponectin-KO mice (compare Figure 2c and 2d with 2g and 2h).
were similar to those of control WT mice (cross-sectional area and number of intraglomerular cells of control KO mice without subtotal nephrectomy, the average cross-sectional area and number of intraglomerular cells in

Figure 1. Representative results of immunohistochemical analyses for adiponectin in non-operated WT (a and c) and WT mice after subtotal nephrectomy (b and d). Control nonoperated mice showed limited glomerular and tubulointerstitial staining for adiponectin (a and c), whereas glomerular and tubulointerstitial immunostaining for adiponectin was augmented after subtotal nephrectomy (b and d). Magnification, ×40 (a and b), ×200 (c and d).

To analyze these changes quantitatively, the glomerular cross-sectional area and number of intraglomerular cells in every specimen were measured (Figure 2i through 2k). Without subtotal nephrectomy, the average cross-sectional area and number of intraglomerular cells of control KO mice were similar to those of control WT mice (cross-sectional area (mm²): 2.55±0.01×10⁻¹³ for KO mice (n=6), 2.52±0.11×10⁻¹³ for WT mice (n=6); intraglomerular cell number (cells/glomerulus): 23.9±0.8 for KO mice, 23.8±1.2 for WT mice; Figure 2i and 2j)]. On the other hand, subtotal nephrectomy resulted in increases in glomerular cross-sectional area and number of intraglomerular cells of both WT (n=6) and KO (n=8) mice at 8 weeks (cross-sectional area: 2.72±0.11×10⁻¹³ and 3.13±0.11×10⁻¹³ mm², respectively, intraglomerular cell number: 34.3±1.0 and 38.3±1.4/section, respectively, Figure 2i and 2j). These increases were more significant in KO mice than in WT mice (P<0.05, for both, Figure 2i and 2j). Furthermore, subtotal nephrectomy resulted in increase area of tubulointerstitial fibrosis in both WT and KO mice (Figure 2k), which was also significantly more severe in KO mice (7.6±1.1%) than in WT mice (4.7±0.3%; P<0.05). Neprhin immunostaining tended to reduce in the remnant kidneys of both WT and KO mice by subtotal renal ablation (supplemental Figure I-a, I-b, I-c, and I-d). To quantify the expression of neprhin, quantitative real-time PCR was performed in the control and remnant kidney. Subtotal nephrectomy resulted in significant reduction of neprhin mRNA levels in the remnant kidney in KO mice than in WT mice (supplemental Figure I-e). Adiponectin deficiency had no effect on neprhin mRNA levels without renal ablation.

**Inflammatory Response in Adiponectin KO Mice and WT Mice**

Next, we investigated glomerular macrophage infiltration, which is regarded as a key event in glomerular injury that leads to renal fibrosis and proteinuria. Immunohistochemical analysis revealed increased number of glomerular infiltration of F4/80-positive macrophages in KO mice after renal ablation (Figure 3a). The gene expressions of F4/80 and CD68, which were specifically expressed in macrophages and macrophage-related cells, were significantly increased in KO mice after subtotal nephrectomy (supplemental Figure II-a). To determine the mechanism of severe glomerular and tubulointerstitial damage in KO mice, we examined the mRNA levels of proteins associated with macrophage infiltration, glomerular and tubulointerstitial fibrosis, oxidative stress, and chronic hypoxia. Subtotal nephrectomy resulted in significant overexpression of VCAM-1, MCP-1, TNF-α, TGF-β1, collagen I, and collagen III mRNA levels in the remnant kidney in KO mice than in WT mice (Figure 3b and 3c). The mRNA levels of NADPH oxidase components, gp91phox, p47phox, and p67phox, were increased in the remnant kidney in KO mice (P<0.05, for both, Figure 3i and 2j). These increases were more significant in KO mice than in WT mice (P<0.05, for both, Figure 3i and 2j). Furthermore, subtotal nephrectomy resulted in increase area of tubulointerstitial fibrosis in both KO and WT mice (supplemental Figure II-b). There were no significant differences in the mRNA levels of VEGF-A, as

### Characteristics of Adiponectin Knockout (KO) and Wild-Type (WT) Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Subtotal Nephrectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (n=6)</td>
<td>KO (n=6)</td>
<td>WT (n=6)</td>
</tr>
<tr>
<td>Body Weight, g</td>
<td>32.7±1.2</td>
<td>31.6±1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>103.0±1.7</td>
<td>103.0±1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, beat/min</td>
<td>681.2±12.9</td>
<td>675.8±20.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of glomeruli/section</td>
<td>184.6±10.9</td>
<td>176.5±7.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood urea nitrogen, mg/dl</td>
<td>29.3±3.6</td>
<td>28.0±1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.11±0.02</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine Clearance, µl/min</td>
<td>208.1±22.0</td>
<td>174.7±35.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum adiponectin, µg/ml</td>
<td>19.0±1.4</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary albumin, mg/Cr</td>
<td>35.2±21.5</td>
<td>27.1±6.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are mean±SEM. P<0.05 compared with WT after subtotal nephrectomy. ND indicates not detectable.
the downstream of hypoxia inductive factor-1α (supplemental Figure II-b).

Adiponectin Supplementation Ameliorates Albuminuria, Glomerular Hypertrophy, and Tubulointerstitial Fibrosis in Subtotal Nephrectomized-KO Mice

To determine the effect of exogenous adiponectin replenishment, KO and WT mice were treated with Ad-APN or Ad-β gal. Four weeks after subtotal nephrectomy, Ad-APN or Ad-β gal was injected intravenously via the tail vein. On day 14 postinjection, plasma adiponectin levels were 65.1±22.9 μg/mL in KO mice treated with Ad-APN (KO/Ad-APN, n=9), not detectable in KO mice treated with Ad-β gal (KO/Ad-β gal, n=9), 59.7±9.2 μg/mL in WT/Ad-APN (n=10), and 19.1±2.0 μg/mL in WT/Ad-β gal (n=9). Immunohistochemical analysis at 4 weeks after Ad-APN injection showed adiponectin accumulation in the glomeruli and interstitium of the remnant kidneys of KO mice (Figure 4a). Such immunohistochemical improvement was coupled with significant decrease in urinary albumin excretion/Cr (mg/g Cr) in KO/Ad-APN compared with KO/Ad-β gal after Figure 2. a through h. Histology of the kidneys of nonoperated WT (a and b), nonoperated KO (c and d), and WT (e and f) and KO (g and h) mice after subtotal nephrectomy. Representative periodic acid-Schiff (PAS)-stained sections (a, c, e, and g) and Masson trichrome stained sections (b, d, f, and h). Note the lack of differences in the glomeruli and tubules between nonoperated WT and KO mice (a and c, b and d). Eight weeks after subtotal nephrectomy, glomerular hypertrophy and tubulointerstitial fibrosis were only modestly increased in WT mice (e and f) but remarkably increased in KO mice (g and h). Mean values of area of glomerular cross-section (i), number of intraglomerular cells (j), and area of interstitial fibrosis (k) were quantitatively analyzed for WT and KO mice with or without subtotal nephrectomy. Magnification, ×200. #P<0.05 for control WT mice. *P<0.05 for renal-ablated WT mice.
Figure 3. a. Immunofluorescence study for F4/80 in glomeruli of nonoperated WT and KO and renal-ablated WT and KO mice. Right panel shows representative F4/80 staining in a glomerulus. Immunohistochemical analysis revealed increased number of glomerular infiltrating F4/80-positive macrophages in KO mice after subtotal nephrectomy. b. The mRNA levels of proteins associated with macrophage infiltration and inflammation. c. The mRNA levels of proteins associated with glomerular and tubulointerstitial fibrosis. d. The mRNA levels of NADPH oxidase components. After subtotal nephrectomy, the mRNA levels of VCAM-1, MCP-1, TNF-α, TGF-β1, collagen I, collagen III, gp91phox, p47phox, and p67phox in the remnant kidney were significantly higher in KO mice than in WT mice. *P<0.05 for control WT mice. **P<0.05 for renal-ablated WT mice.
subtotal nephrectomy (33.9±10.6 mg/g Cr versus 102.3±16.2 mg/g Cr; $P<0.01$, Figure 4b). In contrast, no differences were observed in urine albumin excretion between WT/Ad-β gal and WT/Ad-APN after subtotal nephrectomy (29.7±7.6 mg/g Cr versus 35.5±9.8 mg/g Cr; NS, Figure 4b). Furthermore, Ad-APN-treated subtotal nephrectomized-KO mice had significantly smaller glomerular cross-sectional area (Figures 4c and 5e through 5h), lower number of intraglomerular cells (Figures 4d and 5e through 5h), smaller area of tubulointerstitial fibrosis (Figures 4e and 5e through 5h), lower number of F4/80 positive cells, and lower mRNA levels of F4/80, CD68, VCAM-1, MCP-1, TNF-α, TGF-β1, collagen I, collagen III, gp91phox, p47phox, and p67phox in the remnant kidney (supplemental Figure III), compared with Ad-β gal–treated subtotal nephrectomized-KO mice. In contrast, no differences were observed in those levels between WT/Ad-β gal and WT/Ad-APN after subtotal nephrectomy (Figures 4c through 4e and 5a through 5d; supplemental Figure III).

Discussion

The major findings of the present study are the following: (1) renal injury was associated with accumulation of adiponectin in glomeruli and tubular interstitium; (2) Subtotal nephrectomized-adiponectin KO mice exhibited more severe glomerular hypertrophy, increased number of intraglomerular cells, wider tubulointerstitial fibrosis, lower levels of nephrin mRNA, higher urinary albumin excretion and overexpression of VCAM-1, MCP-1, TNF-α, TGF-β1, collagen I/III, and NADPH oxidase components mRNA levels in the remnant kidney, compared with subtotal nephrectomized-WT mice; (3) Adiponectin treatment ameliorated albuminuria, glomerular hypertrophy, and tubulointerstitial fibrosis, and reduced mRNA levels of VCAM-1, MCP-1, TNF-α, TGF-β1, collagen I, III, NADPH oxidase components in the remnant kidney of subtotal nephrectomized-adiponectin KO mice relative to Ad-β gal-treatment in the same mice.

There is an increasing body of evidence that obesity itself can damage the kidney, even in otherwise healthy subjects. There appears to be an interesting parallel between the effects of obesity and those of diabetes on the kidney. Firstly, increases in renal blood flow and glomerular filtration rate (GFR) have been described in obesity and, secondly, microalbuminuria is reported to be related to obesity. These 2 processes are known to predict future renal dysfunction in diabetes. Experimental and clinical evidence suggests that inflammation and oxidative stress play a role in the pathogenesis of diabetic nephropathy, in addition to, or in concert with, the associated hemodynamic and metabolic changes. Microalbuminuria, defined as urine albumin to urine creatinine ratio of 30 to <300 μg/mg, is an established risk factor for cardiovascular morbidity and mortality in individuals with hypertension and diabetes mellitus and even in healthy subjects. Dysfunction of the vascular endothelium, chronic low-grade inflammation, and oxidative stress are common pathophysiological findings in microalbuminuria and cardiovascular disease. In the present study, subtotal nephrectomy worsened urinary albumin excretion, which was accompanied by glomerular and interstitial changes, although it is difficult to define the abnormal levels of excretion in mice. Mounting evidence indicates that these renal structural abnormalities are a consequence of concerted actions of mechanical stress, caused by glomerular hypertension and hypertrophy, oxidative stress, and inflammatory changes comprising cell infiltration or proliferation and accumulation of extracellular matrix.
pressions of endothelial adhesion molecules and TNF-
IL-10, an antiinflammatory cytokine.3–6 We hypothesized that
human monocyte-derived macrophages through induction of
TIMP-1, which protects vascular wall from plaque rupture, in
macrophages, and selectively increased the expression of
inflammatory markers such as C-reactive protein and IL-6.28 In
ponectinemia is closely associated with increased levels of
matrix.27 Moreover, a causal relationship appears to exist be-
tween these phenomena, because distention of glomerular walls
by intracapillary hypertension could trigger the local
release of reactive oxygen species (ROS) and cytokines and
growth factors.28–30

We and others have reported that adiponectin has antiinflam-
matory and antioxidative properties.7,30,31 Clinically, hypoadi-
ponectinemia is closely associated with increased levels of inflamatory markers such as C-reactive protein and IL-6.28 In
vitro, recombinant adiponectin suppressed TNF-α-induced ex-
pressions of endothelial adhesion molecules and TNF-α in
macrophages, and selectively increased the expression of
TIMP-1, which protects vascular wall from plaque rupture, in
human monocyte-derived macrophages through induction of
IL-10, an antiinflammatory cytokine.3–6 We hypothesized that
the protective effects of adiponectin against renal fibrosis are
mediated by the antioxidative and antiinflammatory effects of
adiponectin. Because our study showed that blood pressure
levels and serum creatinine, creatinine clearance, and blood urea
nitrogen concentrations were not different among WT and KO
mice with or without renal ablation, factors other than hemody-
namics should account for the renal fibrosis in APN-KO mice. In
this regard, adiponectin treatment reversed urinary albumin
excretion and renal fibrosis, which were further upregulated in
KO mice after subtotal nephrectomy compared with WT mice.
These findings clearly show that the lack of adiponectin exac-
erbates renal damage after subtotal nephrectomy, and that
adiponectin accumulation in the remnant kidney may have
protective properties against glomerular and tubulointerstitial
injury via its antiinflammatory and antioxidative effects.

Adipo R1, Adipo R2, and T-cadherin are reported to function
as adiponectin receptors.32,33 Adipo R1 and R2 mediate in-
creased AMP-activated protein kinase, peroxisome proliferators-
activated receptor-α (PPAR-α) ligand activities, and glucose
uptake and fatty-acid oxidation by adiponectin.32 T-cadherin,
which is expressed in endothelium and smooth muscle, has been
identified as an adiponectin-binding protein with preference for
high molecular weight (HMW) adiponectin multimers.33 On the
other hand, the mechanism between adiponectin receptors and
antiinflammatory, antioxidative effects of adiponectin is not
entirely clarified. Recently, it has been reported that adiponectin
protects the organism from systemic inflammation by promoting
the clearance of early apoptotic cells by macrophages.34 This
activity was mediated by calreticulin expressed on the phago-
cytic cell surface and not by any of the previously identified
adiponectin receptors such as Adipo R1, Adipo R2, and
T-cadherin.34 The glomerular infiltrations of macrophages were
significantly increased in KO mice after subtotal nephrectomy in
the present study. It is therefore possible that the increased
inflammation and oxidative stress in KO mice might be medi-
ated by the impaired clearance of early apoptotic cells, although
further studies are necessary to elucidate the precise mechanism.

In conclusion, we demonstrated for the first time accumula-
tion of adiponectin in the injured glomeruli, and that the lack of
adiponectin promotes albuminuria, glomerular hypertrophy and
tubulointerstitial fibrosis in a subtotal nephrectomy mouse
model. Our results suggest that hypoadiponectinemia could
contribute to the exacerbation of renal injury through a proin-
flammatory mechanism and that adiponectin supplementation
might be therapeutically beneficial in renal disorders.

Acknowledgments
We gratefully acknowledge the technical assistance of Mina Sonoda.

Sources of Funding
This work was supported in part by a Grant-in-Aid for Scientific
Research (B) no. 14370327, Grants-in-Aid for Scientific Research on
Priority Areas no. 13137206 and 15081208, Grant-in-Aid for Scientific
Research (B) no. 15390287, Takeda Science Foundation, the Research
Fellowships of the Japan Society for the Promotion of Science for
Young Scientists, Mitsubishi Pharma Research Foundation, Yamanou-
chi Foundation for Research on Metabolic Disorders, The Cell Science
Research Foundation, and Japan Heart Foundation Grant for Research
on Arteriosclerosis Update.

Disclosures
None.

References
1. Gelber RP, Kurth T, Kausz AT, Manson JE, Buring JE, Levey AS,
Gaziano JM. Association between body mass index and CKD in
cDNA cloning and expression of a novel adipose specific collagen-like factor,
15. Gong R, Rifai A, Dworkin LD. Anti-inflammatory effect of hepatocyte
15. Gong R, Rifai A, Dworkin LD. Anti-inflammatory effect of hepatocyte
15. Gong R, Rifai A, Dworkin LD. Anti-inflammatory effect of hepatocyte
15. Gong R, Rifai A, Dworkin LD. Anti-inflammatory effect of hepatocyte
Exacerbation of Albuminuria and Renal Fibrosis in Subtotal Renal Ablation Model of Adiponectin-Knockout Mice

Koji Ohashi, Hirotugu Iwata, Shinji Kihara, Yasuhiko Nakagawa, Noriuyuki Komura, Koichi Fujita, Norikazu Maeda, Makoto Nishida, Fumie Katsube, Ichiro Shimomura, Takahito Ito and Tohru Funahashi

Arterioscler Thromb Vasc Biol. 2007;27:1910-1917; originally published online July 12, 2007;
doi: 10.1161/ATVBAHA.107.147645
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/27/9/1910

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/08/22/ATVBAHA.107.147645.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Figure Legends

**Figure III.** (a) Immunofluorescence study for F4/80 in glomeruli of KO and WT mice treated with Ad-APN or Ad-ß gal. Immunohistochemical analysis revealed decreased number of glomerular infiltrating F4/80-positive macrophages in Ad-APN-treated KO mice than in Ad-ß gal-treated KO mice after subtotal nephrectomy. (b) The mRNA levels of F4/80 and CD68 also significantly decreased in Ad-APN-treated KO mice than in Ad-ß gal-treated KO mice after subtotal nephrectomy. The mRNA levels of proteins associated with macrophage infiltration (c) and glomerular, tubulointerstitial fibrosis (d) and NADPH oxidase components (e) in KO and WT mice treated with Ad-APN or Ad-ß gal. In the renal ablation model, the mRNA levels of VCAM-1, MCP-1, TNF-α, TGF-β1, collagen I and collagen III in the remnant kidney were significantly decreased in Ad-APN-treated KO mice than in Ad-ß gal-treated KO mice, although no significant differences were observed in those mRNA levels between in Ad-ß gal-treated WT mice and in Ad-APN-treated WT mice. #P<0.05 for Ad-ß gal-treated WT mice. *P<0.05 for Ad-ß gal-treated KO mice.
**Supplement Table.** Primers used in RT-PCR protocols.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>36B4</strong></td>
<td>forward 5’-GCTCCAAGCAGATGCAGCA-3’&lt;br&gt;reverse 5’-CCGGATGATGACAGCACAG-3’</td>
</tr>
<tr>
<td>nephrin</td>
<td>forward 5’-AGGAAGGAGAGAGGCTGGAC-3’&lt;br&gt;reverse 5’-CGTTGAGGCGGTAGAAAGAG</td>
</tr>
<tr>
<td>F4/80</td>
<td>forward 5’-CTTTGGCTATGGGCTTCCAGTC-3’&lt;br&gt;reverse 5’-GCAAGGAGACAGAGTTACGTG-3’</td>
</tr>
<tr>
<td>CD68</td>
<td>forward 5’-CTTCTGCTGGAAATGCAA-3’&lt;br&gt;reverse 5’-AGAGGGGCTGGTAGGTTGAT-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>forward 5’-CGGAGTCCGGGCAGGT-3’&lt;br&gt;reverse 5’-GCTGGGTAGAGAATGGATGAACA-3’</td>
</tr>
<tr>
<td>MCP-1</td>
<td>forward 5’-CAGCCAGATGCAGTTAACGC-3’&lt;br&gt;reverse 5’-GCCTACTCATTGTCTCATCTC-3’</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>forward 5’-TGC GAGTCACCATTGTTCTCAT-3’&lt;br&gt;reverse 5’-CATGGTCAGAACGGACTTGGA-3’</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>forward 5’-CACCAGAGACACCTGGATA-3’&lt;br&gt;reverse 5’-TTCAACCCAGGTCTCCTC-3’</td>
</tr>
<tr>
<td>collagen I</td>
<td>forward 5’-GTCCCAACCCCCAAAGAC-3’&lt;br&gt;reverse 5’-CAGCTTCTGAGTTTGGTGATA-3’</td>
</tr>
<tr>
<td>collagen III</td>
<td>forward 5’-TGGTTTCTTCTCACCCTTCTT-3’&lt;br&gt;reverse 5’-TGCAATCCCATCCTGCTACTG-3’</td>
</tr>
<tr>
<td>gp91phox</td>
<td>forward 5’-TTGGGTAGAGGCTCTCTG-3’&lt;br&gt;reverse 5’-TGGGCGGTGTGCAGTGCTATC-3’</td>
</tr>
<tr>
<td>p47phox</td>
<td>forward 5’-GATGGTTCCCATGAGGCG-3’&lt;br&gt;reverse 5’-GGCGGTGTGCAGTGCTATC-3’</td>
</tr>
<tr>
<td>p67phox</td>
<td>forward 5’-GCAATGGCTACTTCCAGAG-3’&lt;br&gt;reverse 5’-CTTATGTTTGAGATGGGAA-3’</td>
</tr>
<tr>
<td>Catalase</td>
<td>forward 5’-CCAGCGAAGATGAAAG-3’&lt;br&gt;reverse 5’-CCACTCTCTCAGGAATCCGC-3’</td>
</tr>
<tr>
<td>Cu,Zn-SOD</td>
<td>forward 5’-CAGCATGGCAGTCTCCAGAG-3’&lt;br&gt;reverse 5’-CACATTGGCCACACGTCCTC-3’</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>forward 5’-TCTCTGGGTGACTTGAGGACA-3’&lt;br&gt;reverse 5’-GTTACAGACGCTGCAAGC-3’</td>
</tr>
</tbody>
</table>
TNF-α: Tumor necrosis factor - α, MCP-1: Monocyte chemoattractant protein-1, VCAM-1: vascular cell adhesion molecule-1, TGF-β1: tumor growth factor-β1, Cu-Zn-SOD: Cu,Zn-superoxide dismutase, VEGF-A: vascular endothelial growth factor-A
Fig. 1

(a) Image of tissue sample

(b) Image of tissue sample

(c) Image of tissue sample

(d) Image of tissue sample

(e) Bar graph showing the expression of Nephrin/36B4 mRNA in WT and KO mice under Control and Nephrectomy conditions. The graph indicates significant differences (* and #) between groups.
Supplemental Figure Legends

Figure II. (a) The mRNA levels of F4/80 and CD68 as macrophage marker. (b) The mRNA levels of Catalase, Cu, Zn-SOD and VEGF-A. Following subtotal nephrectomy, no significant differences were observed in mRNA levels of Catalase, Cu, Zn-SOD and VEGF-A between KO and WT mice. #P<0.05 for control WT mice. *P<0.05 for renal-ablated WT mice.
Supplemental Figure Legends

**Figure I.** (a – d) Representative results of immunohistochemical analyses for nephrin in non-operated WT (a), KO (b) mice and operated WT (c), KO (d) mice after subtotal nephrectomy. Nephrin immuno-staining tended to reduce in the remnant kidneys of both WT and KO mice by subtotal renal ablation (a - d). Nephrin mRNA levels of non-operated WT, non-operated KO, and WT, KO mice after subtotal nephrectomy (e). Subtotal nephrectomy resulted in significant reduction of nephrin mRNA levels in the remnant kidney in KO mice than in WT mice. #P<0.05 for control WT mice. *P<0.05 for renal-ablated WT mice.
Fig. III

a) F4/80 positive cells (glm)

b) CD68

c) VCAM1

d) MCP-1

e) Collagen III

WT vs. KO: β-gal APN vs. β-gal APN

* indicates significant difference compared to WT

# indicates significant difference compared to KO