



# Adiponectin regulates albuminuria and podocyte function in mice

Kumar Sharma,<sup>1,2,3</sup> Satish RamachandraRao,<sup>1,2,3</sup> Gang Qiu,<sup>3</sup> Hitomi Kataoka Usui,<sup>3,4</sup> Yanqing Zhu,<sup>3</sup> Stephen R. Dunn,<sup>3</sup> Raogo Ouedraogo,<sup>5</sup> Kelly Hough,<sup>5</sup> Peter McCue,<sup>6</sup> Lawrence Chan,<sup>7</sup> Bonita Falkner,<sup>8</sup> and Barry J. Goldstein<sup>5</sup>

<sup>1</sup>Translational Research in Kidney Disease, Division of Nephrology, Department of Medicine, University of California, San Diego, La Jolla, California, USA. <sup>2</sup>Veterans Administration San Diego Healthcare System, La Jolla, California, USA.

<sup>3</sup>Center for Novel Therapies in Kidney Disease, Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania, USA.

<sup>4</sup>Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, Okayama, Japan. <sup>5</sup>Division of Endocrinology, Diabetes, and Metabolic Diseases, Department of Medicine, and <sup>6</sup>Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania, USA. <sup>7</sup>Division of Endocrinology, Diabetes, and Metabolic Diseases, Baylor College of Medicine, Houston, Texas, USA.

<sup>8</sup>Center for Hypertension, Division of Nephrology, Thomas Jefferson University, Philadelphia, Pennsylvania, USA.

**Increased albuminuria is associated with obesity and diabetes and is a risk factor for cardiovascular and renal disease. However, the link between early albuminuria and adiposity remains unclear. To determine whether adiponectin, an adipocyte-derived hormone, is a communication signal between adipocytes and the kidney, we performed studies in a cohort of patients at high risk for diabetes and kidney disease as well as in adiponectin-knockout (*Ad*<sup>-/-</sup>) mice. Albuminuria had a negative correlation with plasma adiponectin in obese patients, and *Ad*<sup>-/-</sup> mice exhibited increased albuminuria and fusion of podocyte foot processes. In cultured podocytes, adiponectin administration was associated with increased activity of AMPK, and both adiponectin and AMPK activation reduced podocyte permeability to albumin and podocyte dysfunction, as evidenced by zona occludens-1 translocation to the membrane. These effects seemed to be caused by reduction of oxidative stress, as adiponectin and AMPK activation both reduced protein levels of the NADPH oxidase Nox4 in podocytes. *Ad*<sup>-/-</sup> mice treated with adiponectin exhibited normalization of albuminuria, improvement of podocyte foot process effacement, increased glomerular AMPK activation, and reduced urinary and glomerular markers of oxidant stress. These results suggest that adiponectin is a key regulator of albuminuria, likely acting through the AMPK pathway to modulate oxidant stress in podocytes.**

## Introduction

Chronic kidney disease and albuminuria have recently been recognized as among the most significant clinical risk factors for cardiovascular disease (CVD), hospitalization, and all-cause mortality (1–5). Microalbuminuria (albumin/creatinine ratio [ACR], 30–300 µg/mg), resulting from leakage of albumin across the glomerular podocyte filtration barrier into the urine, is considered a marker for dysfunction of the systemic vasculature and indicates a heightened risk of CVD (1, 2, 6). Recently, albuminuria in the so-called high normal range (ACR, 10–30 µg/mg) has been identified as a risk factor for CVD (7). Renal dysfunction may contribute to overall CVD by promoting vascular thickening and vascular calcification (8) as well as by activating inflammatory pathways (9). It has also been recognized that insulin resistance is closely associated with oxidant stress, early decline in renal function, and albuminuria (1, 10). Despite the close relationships demonstrated to exist between CVD and kidney dysfunction, the mechanism linking these entities has not yet been elucidated.

**Nonstandard abbreviations used:** AA, African American; ACR, albumin/creatinine ratio; AdipoR, adiponectin receptor; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; ARA, adenine 9-β-D-arabinofuranoside; CVD, cardiovascular disease; fAd, full-length adiponectin; gAd, globular adiponectin; Nox, NADPH oxidase; 8-OHdG, 8-hydroxydeoxyguanosine, PAI-1, plasminogen activator inhibitor-1; ZO-1, zonula occludens-1.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Citation for this article:** *J. Clin. Invest.* 118:1645–1656 (2008). doi:10.1172/JCI32691.

Adiponectin, a 30-kDa circulating plasma protein primarily secreted by adipocytes, has been recently recognized to be a key predictive factor for cardiovascular mortality in patients with renal dysfunction (9, 11). Adiponectin is found at relatively high total levels in the bloodstream of human subjects, ranging in concentration from 5 to 30 µg/ml. Adiponectin is secreted from adipose tissue and circulates in multimeric forms ranging from trimers to high-molecular weight oligomers containing 12- to 18-mers (12). Interestingly, a recombinant fragment of adiponectin containing the C-terminal globular head domain interacts with cellular adiponectin receptors and mimics many of the actions of the full-length, oligomeric form of the protein (12). The globular domain can be generated by cleavage from the full-length protein by neutrophil elastase and other proteases (13, 14), although it remains controversial whether the globular fragment of adiponectin is generated in situ or circulates in vivo. Both forms of adiponectin exert largely beneficial effects to improve insulin sensitivity and decrease the adverse effects of inflammatory mediators in vascular cells (12, 15). The globular and full-length forms of adiponectin (gAd and fAd, respectively) both bind to 2 adiponectin receptors (AdipoR1 and AdipoR2) (16) and signal via stimulation of 5'-AMP activated protein kinase (AMPK) and potentially other intracellular pathways (16, 17). Protective effects of adiponectin may involve reduction of oxidant stress, possibly by inhibition of NADPH oxidases, as our group has shown in endothelial cells (17) and in myocardial tissue (18). Plasma adiponectin levels are reduced with increasing



**Table 1**  
Characteristics of AA subjects

Characteristic	Value (mean ± SD)
Age, yr	40 ± 4.0
Sex, M/F	4/16
BMI	40.14 ± 7.10
SBP, mmHg	128 ± 13
DBP, mmHg	73 ± 7
MAP, mmHg	91 ± 8
Total cholesterol, mg/dl	176 ± 47
HDL, mg/dl	43 ± 10
LDL, mg/dl	112 ± 43
Triglycerides, mg/dl	102 ± 36
M/I	5.24 ± 2.72
Urine ACR, µg/mg (median)	8.96 ± 7.01 (7.18)
Plasma adiponectin, ng/ml (median)	12.0 ± 5.1 (12.8)
Plasma IL-6, pg/ml (median)	7.0 ± 14 (3.2)
Plasma PAI-1, ng/ml (median)	94.4 ± 37.1 (99.4)

SBP, systolic blood pressure; DBP, diastolic blood pressure; M/I, insulin sensitivity index during the insulin clamp (calculated as insulin-stimulated glucose uptake [M] divided by the steady-state insulin concentration [I]).

visceral obesity and tightly correlate with insulin resistance and development of type 2 diabetes mellitus (19). Interestingly, in patients with type 1 diabetes, a SNP in the adiponectin promoter showed linkage with diabetic nephropathy (20), and low plasma adiponectin levels were predictive of the development of coronary artery calcification (21, 22), which suggests an important role for adiponectin in development of kidney and macrovascular disease with hyperglycemia.

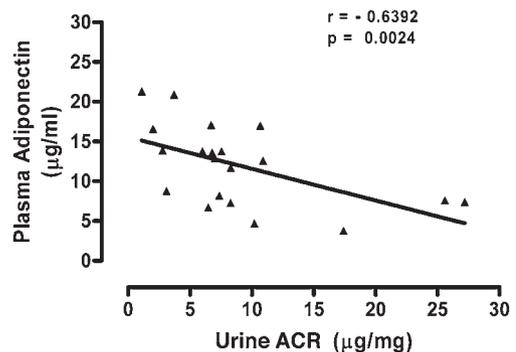
In the African-American (AA) population, low plasma adiponectin levels have been reported in obese subjects and may be predictive of the development of type 2 diabetes (23, 24). Of note, both diabetes (25) and AA ethnicity are strong predisposing risk factors for progressive kidney disease (26). Although both adiponectin levels and albuminuria are associated with CVD and kidney dysfunction, previous studies attempting to link adiponectin with albuminuria have been inconclusive (27–29). To our knowledge, a causative role for adiponectin in the development of albuminuria in its early stages has not previously been demonstrated.

In the present study, we found that circulating adiponectin levels had a strong negative correlation with the degree of albuminuria in nondiabetic obese AA subjects. In the adiponectin knockout (*Ad<sup>-/-</sup>*) mouse, urinary levels of albumin and hydrogen peroxide were increased, and podocyte foot process effacement was evident. In cell culture studies with podocytes, adiponectin potently decreased permeability to albumin and induced translocation of zona occludens-1 (ZO-1) to the plasma membrane, largely via an AMPK-dependent pathway. In addition, adiponectin reduced the renal predominant NADPH oxidase Nox4 (30) in podocytes. Treatment of *Ad<sup>-/-</sup>* mice with exogenous adiponectin decreased urine albumin and urinary hydrogen peroxide in association with a marked improvement in podocyte morphology, increased glomerular AMPK activity, and reduced glomerular Nox4. Diabetic *Ad<sup>-/-</sup>* mice also had marked increases in albuminuria that were reduced by exogenous adiponectin. Our data thus provide what we believe to be the first evidence that podocytes are a direct target of adipocyte action.

**Results**

*Low adiponectin levels correlate with albuminuria in obese AAs.* A total of 20 obese AA subjects who were on no medications for lipid or glycemic control were examined for relationships between ACR and adipocytokines. Subject characteristics are presented in Table 1. Serum creatinine was normal ( $\leq 1.4$  mg/dl) and ACR was below 30 µg/mg in all subjects. In this sample of obese subjects, there was a statistically significant negative correlation between plasma adiponectin concentration and urinary albumin excretion, expressed as log-transformed urine ACR ( $r = -0.639$ ,  $P < 0.01$ ; Figure 1 and Table 2). Of the other variables (Table 2), there was a significant correlation between ACR and HDL by the Spearman rank-sum test ( $r = -0.501$ ;  $P = 0.024$ ), but the correlation did not reach significance by Pearson linear regression ( $P = 0.054$ ). No significant correlations between ACR and plasma levels of IL-6 or plasminogen activator inhibitor-1 (PAI-1) were found. There were no significant correlations between ACR and other clinical parameters including BMI, insulin sensitivity, blood pressure, total cholesterol, LDL, and triglycerides.

*Increased albuminuria and oxidant stress in *Ad<sup>-/-</sup>* mice.* In order to determine whether adiponectin may be a causative factor for albuminuria, we examined the *Ad<sup>-/-</sup>* mouse. This mouse has been previously reported to have normal glucose tolerance and insulin sensitivity when fed normal rodent chow (31). The *Ad<sup>-/-</sup>* mice had normal levels of blood pressure, blood glucose, insulin, and lipids as well as normal body weight (data not shown). The degree of albuminuria in the *Ad<sup>-/-</sup>* mice increased more than 2-fold between 1 and 3 months of age and increased further at 4 months of age compared with age- and sex-matched WT mice ( $106.2 \pm 12.9$  versus  $31.3 \pm 1.8$  µg albumin/mg creatinine;  $P < 0.01$ ; Figure 2A). Mice with diabetes were also examined. Diabetes induced with multiple low doses of streptozotocin in WT C57BL/6 mice only modestly increased albuminuria, even 4 months after induction (Figure 2B). The degree of hyperglycemia and body weight was similar in WT and *Ad<sup>-/-</sup>* diabetic mice at 4 months of diabetes (blood glucose,  $495 \pm 128$  versus  $522 \pm 135$  mg/dl; body weight,  $27.2 \pm 2.7$  versus  $27.3 \pm 2.3$  g). However, induction of type 1 diabetes in *Ad<sup>-/-</sup>* mice led to a significant increase in albuminuria within 2 months of diabetes induction and exhibited a progressive increase at 4 months after diabetes induction (Figure 2B).



**Figure 1**  
Negative correlation between albuminuria and plasma adiponectin levels in obese AAs. Data show regression between adiponectin levels and urine ACRs. Confidence intervals, Spearman’s correlation coefficient, and P values for all variables tested are listed in Table 2.



**Table 2**  
Association between selected variables and urine ACR

Variable	Linear regression			Spearman rank-sum	
	<i>r</i>	95% CI	<i>P</i> <sup>A</sup>	<i>r</i>	<i>P</i> <sup>A</sup>
Adipo	-0.639	-0.843 to -0.274	0.002	-0.595	0.005
Age	-0.027	-0.464 to 0.419	0.906	-0.064	0.906
BMI	0.312	-0.150 to 0.663	0.179	0.389	0.089
SBP	0.161	-0.302 to 0.556	0.496	0.165	0.484
DBP	0.244	-0.222 to 0.620	0.299	0.141	0.552
T.chol	-0.080	-0.505 to 0.375	0.734	-0.258	0.270
HDL	-0.435	-0.736 to 0.054	0.054	-0.501	0.024
LDL	-0.026	-0.463 to 0.421	0.913	-0.159	0.502
Trig	-0.231	-0.234 to 0.611	0.325	0.030	0.899
IL-6	0.059	-0.439 to 0.489	0.802	0.327	0.159
PAI-1	0.230	-0.236 to 0.610	0.329	0.102	0.669
M/I	-0.436	0.750 to 0.038	0.070	-0.362	0.139

CI, confidence interval; Adipo, adiponectin; SBP, systolic blood pressure; DBP, diastolic blood pressure; T.chol, total cholesterol; Trig, triglycerides; M/I, insulin sensitivity index during the insulin clamp (calculated as insulin-stimulated glucose uptake [M] divided by the steady-state insulin concentration [I]).<sup>A</sup>*P* values are 2-tailed.

Because oxidant stress is a close accompaniment to the development of cardiovascular complications in states of adiponectin deficiency and may be regulated by adiponectin (17, 18), urinary levels of hydrogen peroxide were measured. Urinary hydrogen peroxide was chosen as a measure of oxidant stress because it is relatively stable, is present at high concentrations in the urine, and reflects both systemic and renal oxidant stress (32, 33). Urinary hydrogen peroxide levels increased in *Ad*<sup>-/-</sup> mice (Figure 2C). With the additional stress of hyperglycemia, there was a marked increase in urinary levels of hydrogen peroxide (Figure 2C).

**Direct effects of adiponectin on podocytes.** To determine how adiponectin may contribute to albuminuria, we examined EM sections of the glomeruli from WT and *Ad*<sup>-/-</sup> mice at 3 months of age. Podocyte foot processes were segmentally fused in the *Ad*<sup>-/-</sup> glomeruli (Figure 2D). Glomerular basement membrane thickness, endothelial cells, and mesangial cells were similar in appearance to those of normal WT mice. Thus adiponectin deficiency is associated with podocyte dysfunction.

To determine whether adiponectin had direct effects on podocyte function, a permeability assay was used to measure albumin permeability across a differentiated podocyte cell monolayer *in vitro*. Compared with the degree of albumin permeability across podocytes cultured on porous membranes in serum-free conditions without adiponectin, permeability was significantly reduced with the addition of gAd or fAd (Figure 3A). These data indicate a direct action of adiponectin on podocytes independent of the systemic and/or metabolic effects of adiponectin. As determined by real-time PCR, AdipoR1 of kidney and podocytes was expressed to a similar degree as in liver, but AdipoR2 was much reduced in kidney and podocytes compared with liver (Figure 3, B and C). The reduction of AdipoR2 protein in kidney and podocytes compared with liver was demonstrated by immunoblotting (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI32691DS1).

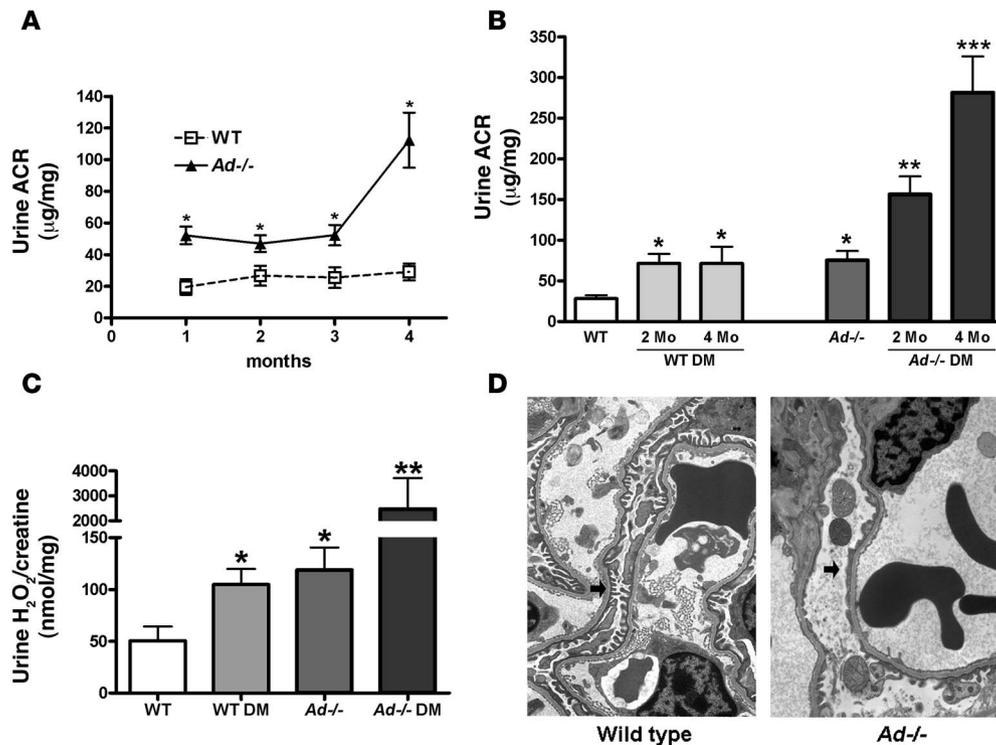
**Adiponectin stimulates AMPK in podocytes.** Among the pathways implicated in adiponectin action, the AMPK pathway appears to have a major role for adiponectin's activity in liver and skeletal

muscle as well as its protective effect in cardiomyocytes (34). The baseline AMPK activity in podocytes cultured in normal glucose increased with addition of adiponectin (Figure 4, A-C). AMPK $\alpha$  phosphorylation was further reduced by high glucose exposure, but the reduction was prevented by adiponectin (Figure 4, A-C). A functional role for AMPK was demonstrated by a specific activator of AMPK, 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR), which reduced permeability of podocytes to albumin, similar to the effect of adiponectin (Figure 4D). Furthermore, a specific inhibitor of AMPK, adenine 9- $\beta$ -D-arabinofuranoside (ARA), increased permeability to albumin, either alone or in the presence of adiponectin (Figure 4D).

To determine whether podocyte proteins associated with podocyte dysfunction are affected by adiponectin, we used ZO-1, a tight junction protein that is highly expressed adjacent to the insertion of the slit diaphragm of the foot process (35). ZO-1 links slit diaphragm proteins through its PDZ (PSD-95/disc-large/ZO-1) domains to the actin cytoskeleton and is translocated away from the plasma membrane to the cytosol in response to high glucose exposure (36) and to puromycin (37). As shown by confocal analysis, ZO-1 was markedly redistributed to the cytosol with high glucose exposure or with AMPK inhibition by ARA (Figure 5). Treatment of podocytes with adiponectin or AICAR restored the linear membrane localization of ZO-1 (Figure 5, A-C).

**Adiponectin replacement restores normoalbuminuria.** To determine whether adiponectin replacement is sufficient to treat increased urinary levels of albumin, gAd or fAd was administered to *Ad*<sup>-/-</sup> mice at 4 months of age, after the onset of increased albuminuria and foot process effacement. Adiponectin administration normalized albuminuria in *Ad*<sup>-/-</sup> mice (Figure 6A). To determine whether exogenous adiponectin affects albuminuria in the clinically relevant condition of diabetes, fAd was administered to mice 2 months after diabetes induction. As shown in Figure 6A, the treated *Ad*<sup>-/-</sup> diabetic mice had a marked reduction in albuminuria after 10 days of adiponectin treatment.

A role for AMPK was also demonstrated, as AICAR administration restored albuminuria in *Ad*<sup>-/-</sup> mice (Figure 6A). Adiponectin administration significantly restored podocyte foot



**Figure 2**

*Ad*<sup>-/-</sup> mice exhibit increased albuminuria, oxidant stress, and podocyte dysfunction. **(A)** Urine ACR in *Ad*<sup>-/-</sup> mice significantly increased compared with corresponding age-matched WT mice at 1, 2, 3, and 4 months of age (*n* = 10 per group). \**P* < 0.01 versus corresponding age-matched WT. **(B)** WT and *Ad*<sup>-/-</sup> mice were made diabetic with low-dose streptozotocin, and urine ACR was measured before and at 2 and 4 months of diabetes. Albuminuria was significantly increased in *Ad*<sup>-/-</sup> mice with diabetes compared with corresponding WT diabetic groups (*n* = 5–10 per group). DM, diabetes mellitus. \**P* < 0.05 versus WT control; \*\**P* < 0.05 versus WT DM at 2 mo; \*\*\**P* < 0.05 versus WT DM at 4 mo. **(C)** Urinary hydrogen peroxide/creatinine levels significantly increased in *Ad*<sup>-/-</sup> mice with and without diabetes (*n* = 10 per group). \**P* < 0.05 versus WT control; \*\**P* < 0.05 versus WT DM at 2 mo. Values are mean ± SEM. **(D)** Podocyte foot processes were segmentally effaced in *Ad*<sup>-/-</sup> mouse kidneys by EM. Arrows denote areas of normal foot processes in WT kidneys and areas of foot process effacement in *Ad*<sup>-/-</sup> glomeruli. Images are representative of 10 EM images per kidney from 2 mice per group. Original magnification, ×5,000.

processes in *Ad*<sup>-/-</sup> mice (Figure 6, B and C). AMPK activity in glomeruli was measured with an antibody to p-AMPK and found to be present primarily in podocytes of glomeruli of WT mice (Figure 6D). AMPK activity was reduced in *Ad*<sup>-/-</sup> mouse glomerular cells and improved with adiponectin treatment (Figure 6, E and F).

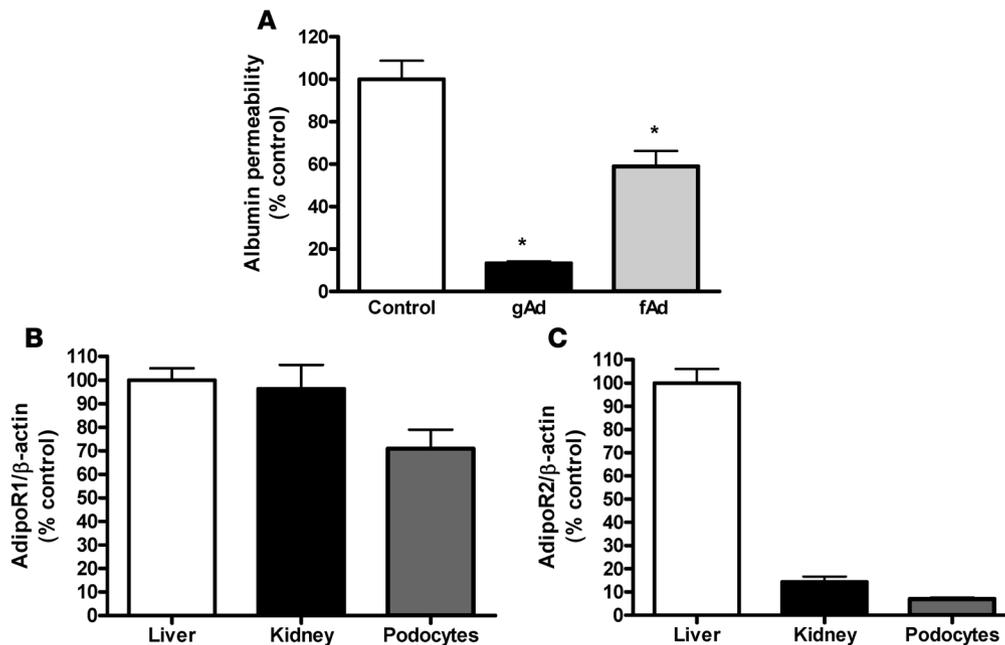
**Role of Nox4 in reduction of oxidant stress by adiponectin.** Because oxidant stress has been linked to podocyte dysfunction and albuminuria (38), we sought to determine whether exogenous adiponectin regulates oxidant stress in the *Ad*<sup>-/-</sup> mice and evaluated the source of oxidant production. Increased urinary levels of hydrogen peroxide in 4-month-old *Ad*<sup>-/-</sup> mice was reduced with treatment with gAd, fAd, or AICAR (Figure 7A). Furthermore, glomerular 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine were increased in *Ad*<sup>-/-</sup> mice and were restored with gAd treatment (Figure 7, B and C). Nox4, one of the several NADPH oxidases that have been cloned, is expressed in a variety of tissues, with the highest expression being in the kidney. As shown by real-time PCR, Nox4 was significantly increased in *Ad*<sup>-/-</sup> kidneys and was reduced to control levels with gAd treatment (Supplemental Figure 2). Nox1 and Nox2 were expressed at low levels in the kidney and did not increase in *Ad*<sup>-/-</sup> mice. As shown by immunofluorescence with double staining, glomeru-

lar Nox4 was clearly present in podocytes of WT kidneys (Figure 7D) as well as in other glomerular and tubular cells. Glomerular Nox4 was increased in *Ad*<sup>-/-</sup> kidneys and was reduced with gAd treatment (Figure 7, E and F).

Nox4 protein was evident in podocyte cell culture grown in serum-free conditions and further increased with high glucose exposure (Figure 8, A, B, and D). As shown by confocal analysis, podocyte Nox4 was primarily perinuclear and at the cell periphery (Figure 8B). Addition of adiponectin was sufficient to suppress podocyte Nox4 (Figure 8, A, B, and D). Furthermore, the AMPK activator AICAR reduced Nox4 protein levels to a similar degree as did adiponectin (Figure 8, C and D).

**Discussion**

In the present study, we report that plasma adiponectin level was inversely correlated with urinary albumin excretion in obese AAs. *Ad*<sup>-/-</sup> mice had increased levels of albuminuria and increased podocyte dysfunction, which indicates that adiponectin deficiency contributes to altered permeability, likely via podocyte dysfunction. AMPK regulation by adiponectin appears to contribute to the mechanism of podocyte dysfunction, because protective effects of adiponectin on podocyte permeability are blocked by AMPK inhibition and mimicked by an

**Figure 3**

Adiponectin inhibits permeability across a podocyte monolayer. (A) Permeability of albumin across a podocyte monolayer was reduced by gAd or fAd at 3  $\mu\text{g/ml}$  ( $n = 3$  per group). Cells were treated as described in Methods, and permeability was assessed by albumin concentration across podocyte monolayer. Values (mean  $\pm$  SEM) are presented as percent of control. \* $P < 0.01$  versus control. (B and C) Expression of AdipoR1 (B) and AdipoR2 (C) by real-time PCR in WT mouse liver, kidney, and differentiated podocytes. Values (mean  $\pm$  SEM) are presented relative to  $\beta$ -actin and expressed as 100% in mouse liver.

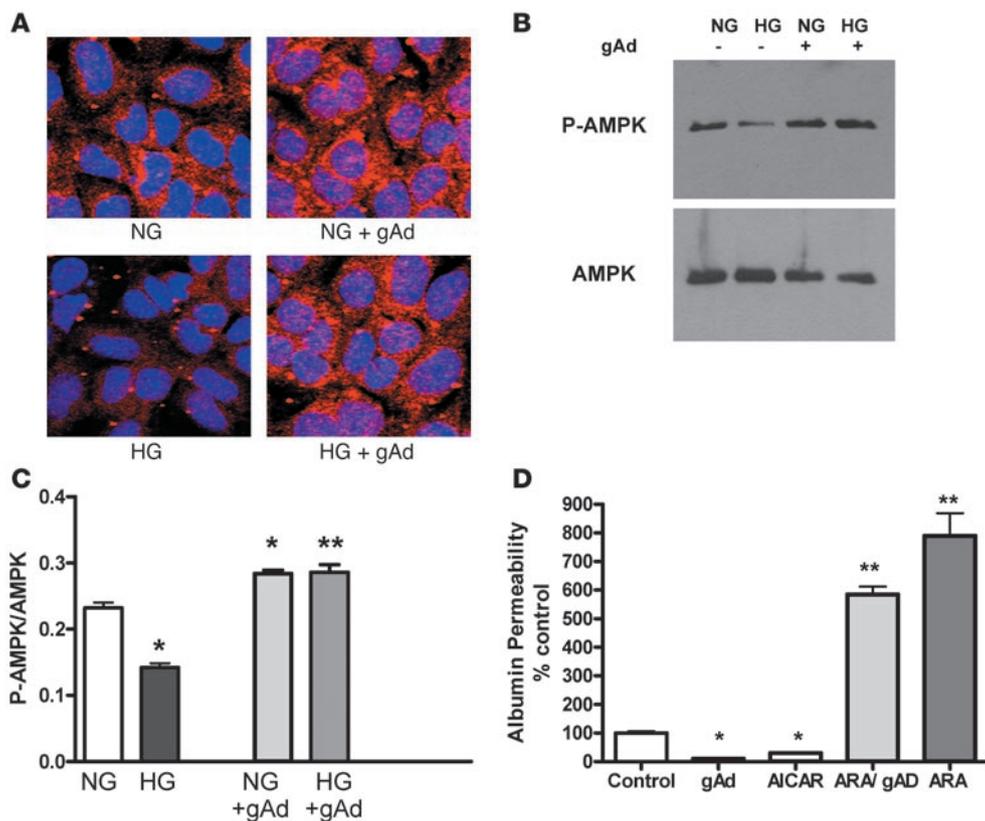
AMPK activator. Additionally, Nox4 is present in podocytes, is regulated by adiponectin, and may contribute to podocyte dysfunction. Administration of adiponectin to  $Ad^{-/-}$  mice normalized albuminuria and oxidant stress, improved podocyte foot processes, increased glomerular AMPK activity, and reduced glomerular Nox4.

AAs have a disproportionate and excessive representation of end-stage renal disease (39). AAs also have high rates of obesity, which heightens risk for kidney disease and CVD. Low adiponectin levels have been identified in obese AAs and are also associated with susceptibility to diabetes (23, 24). A relationship between low adiponectin levels and albuminuria has been reported in males with essential hypertension (28) and in Japanese males and females with obesity (40). To our knowledge, our study is the first to link low adiponectin levels with albuminuria in the obese AA population. This correlation was noted in a population before the onset of diabetes and overt renal dysfunction. The degree of albuminuria in our cohort was within the so-called normal range and thus represents a very early manifestation of kidney disease in association with obesity and insulin resistance. The main conclusion supported by the clinical data is that low adiponectin levels are tightly correlated to this early rise in albuminuria.

Chronic kidney disease is a strong risk factor for CVD mortality (1, 3–5). To some degree, the increased risk of mortality and CVD in chronic kidney disease may be explained by levels of adipokines, including elevated levels of proinflammatory adipokines and reduced adiponectin (9, 11). Adiponectin reduction has been well documented in states of obesity and prediabetes (41), conditions often associated with microalbuminuria

(10). It should be noted that subsequent to the development of overt proteinuria and renal insufficiency (27, 29) there is a reported increase in plasma adiponectin levels. However, several independent groups have reported that a region of chromosome 3q contains a susceptibility locus for diabetic nephropathy in patients with both type 1 (42, 43) and type 2 diabetes (44, 45), and one group evaluated 14 candidate genes on chromosome q and found the strongest linkage with a SNP for the promoter of adiponectin (20). Our present findings of a negative correlation between adiponectin and low levels of albuminuria in patients, a moderate increase in albuminuria in adiponectin-deficient mice, and a dramatic increase in the degree of albuminuria in diabetic mice as a result of adiponectin deficiency point to an important role for adiponectin in the initial development of increased albuminuria.

A vascular protective role of AMPK has been demonstrated in several cell types (17, 46, 47). Our present study demonstrated that AMPK plays a critical role in permeability of podocytes and that AMPK activity in podocytes is regulated by adiponectin. AMPK is a heterotrimeric signaling kinase and a critical energy-sensing pathway with important functions to stimulate glucose uptake. It has previously been demonstrated that the effect of adiponectin on various cell types involves AMPK as well as other pathways, such as the PPAR (48, 49) and cAMP-PKA pathways (17, 47). A recent study using rat glomerular epithelial cells demonstrated that AMPK contributes to high glucose exposure-induced cell hypertrophy (50). ZO-1 localization along the membrane is associated with tight junction adherence and normal function of the slit diaphragm. Adiponectin and AMPK activation promoted ZO-1 membrane localization,



**Figure 4**

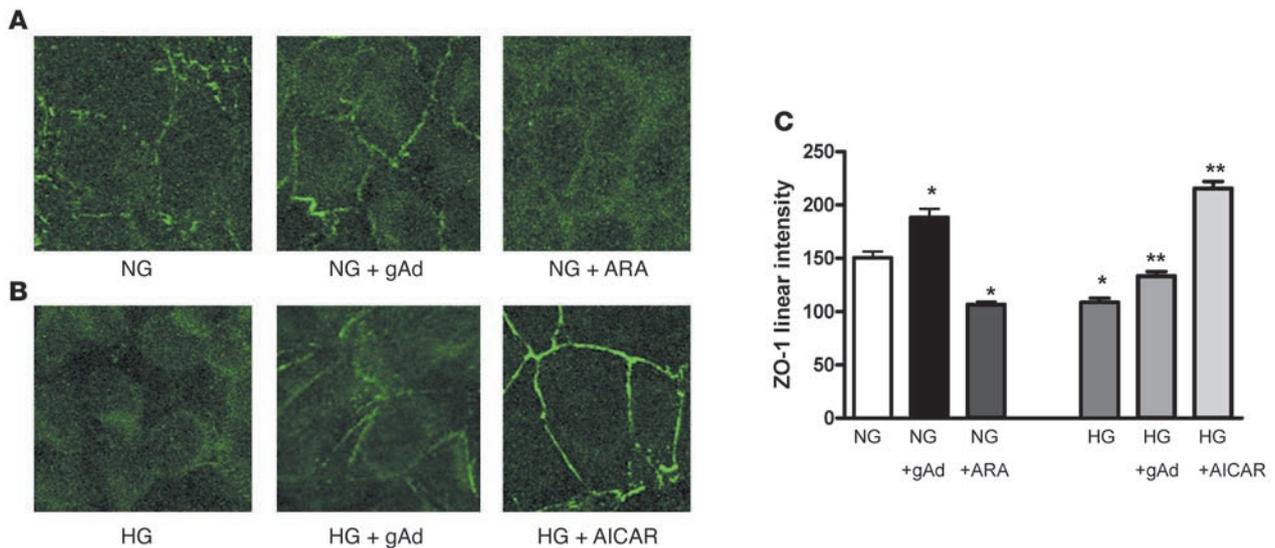
AMPK activity is increased by adiponectin and regulates podocyte permeability. (A and B) Treatment of podocytes with 3  $\mu$ g/ml gAd for 24 h increased AMPK activity in podocytes cultured in normal glucose (NG; 5.5 mM D-glucose) and high glucose (HG; 25 mM D-glucose), as demonstrated by confocal microscopy (A) and immunoblotting (B). AMPK activity was assessed with antibodies specific for the p-AMPK $\alpha$  subunit. Total AMPK $\alpha$  was measured with antibody for AMPK $\alpha$  as a loading control (B). Images are representative confocal photographs and immunoblots from 5 separate experiments. (C) Quantitation of P-AMPK $\alpha$ /AMPK from immunoblots in B ( $n = 5$ ). Values are mean  $\pm$  SEM. \* $P < 0.05$  versus normal glucose; \*\* $P < 0.05$  versus high glucose alone. (D) Albumin permeability was decreased by the AMPK activator AICAR (1 mM) and increased by the AMPK inhibitor ARA ( $n = 5$  per group). The effect of adiponectin to reduce permeability was also blocked by ARA. Cells were treated as described in Figure 3A and Methods. Values (mean  $\pm$  SEM) are presented as percent of control. \* $P < 0.01$  versus control; \*\* $P < 0.001$  versus gAd alone.

which suggests that ZO-1 may be a direct target protein for AMPK. Because the podocyte is the major cell type protecting the glomerulus from leaking albumin into the urinary space, additional podocyte proteins regulated by AMPK will be of major interest and are likely to be involved in linking obesity, hypoadiponectinemia, and albuminuria.

One potential pathway by which adiponectin and AMPK activation may provide protection against albuminuria and podocyte permeability is via reduction of oxidant stress (17, 18, 51). Nox4 is a recently described nonphagocytic NADPH oxidase that is highly expressed in the kidney (30). Our present results demonstrated that podocytes expressed Nox4 and that adiponectin and AMPK regulated Nox4 protein in podocytes. Oxidant stress has been consistently linked with insulin resistance, obesity, and adiponectin deficiency. The kidney's contribution to oxidant stress has been largely ignored in settings of insulin resistance. Our results demonstrated that systemic adiponectin deficiency caused upregulation of Nox4 in the kidney and podocytes, thus providing another critical link among obesity, insulin resistance, and oxidant stress. Of note, Ohashi et al. used a different adiponectin-deficient mouse and found

that adiponectin deficiency accentuates albuminuria in the 5/6 nephrectomy model (52). Thus it is likely that adiponectin deficiency may be a critical risk factor in a variety of kidney diseases associated with albuminuria.

Our results suggest that several approaches may be successful in lowering the development of microalbuminuria and possibly CVD in at-risk populations. Identification of low adiponectin levels and increased albuminuria likely identifies a high risk profile with regard to kidney disease and CVD. In addition to obese AAs and obese Japanese (40), it is likely that similar findings will be made in other ethnic populations with obesity and/or insulin resistance (53) as well as type 1 diabetes (21, 22). Maneuvers to raise adiponectin levels, such as weight reduction, renin-angiotensin system blockade (54), and PPAR $\gamma$  agonists (48), may be beneficial for renal and potentially cardiovascular protection in at-risk populations. Treatment with metformin may be useful, because metformin raises AMPK activity independent of adiponectin (55). Inhibition of specific NADPH oxidase isoforms, such as Nox4, will likely reduce podocyte dysfunction in states of adiponectin deficiency and has previously been shown to benefit diabetic nephropathy in a rat model (56). Treatment



**Figure 5**

ZO-1 localization is regulated by adiponectin and AMPK in podocytes. (A) Immunofluorescence microscopy demonstrated linear ZO-1 staining along the cell membranes of podocytes with normal glucose exposure, which was further enhanced by treatment with gAd and markedly reduced with inhibition of AMPK by ARA. (B) High glucose exposure-induced reduction of linear staining of ZO-1 was attenuated by adiponectin and increased with AMPK activation by AICAR. (C) Semiquantitation of the data from 30 cells per coverslip for each condition in A and B. Experiments were repeated 5 times and expressed as mean  $\pm$  SEM of linear staining per condition. \* $P < 0.05$  versus normal glucose; \*\* $P < 0.05$  versus high glucose.

with gAd or fAd is another potentially attractive option to treat podocyte dysfunction and albuminuria. It is possible that these approaches will only be successful in early-stage kidney disease, when podocyte function will be responsive to AMPK, and before there is widespread podocyte depletion, as noted in situations of severe proteinuria (57).

In summary, circulating adiponectin levels are inversely related to albuminuria in obese AAs without diabetes or overt kidney disease. Adiponectin plays a protective role to reduce albuminuria by directly affecting podocyte function via the AMPK pathway. Our results provide a strong pathobiologic rationale to intervene in the adiponectin-AMPK-Nox4 pathway to protect against albuminuria and potentially affect early renal disease as well as associated CVD.

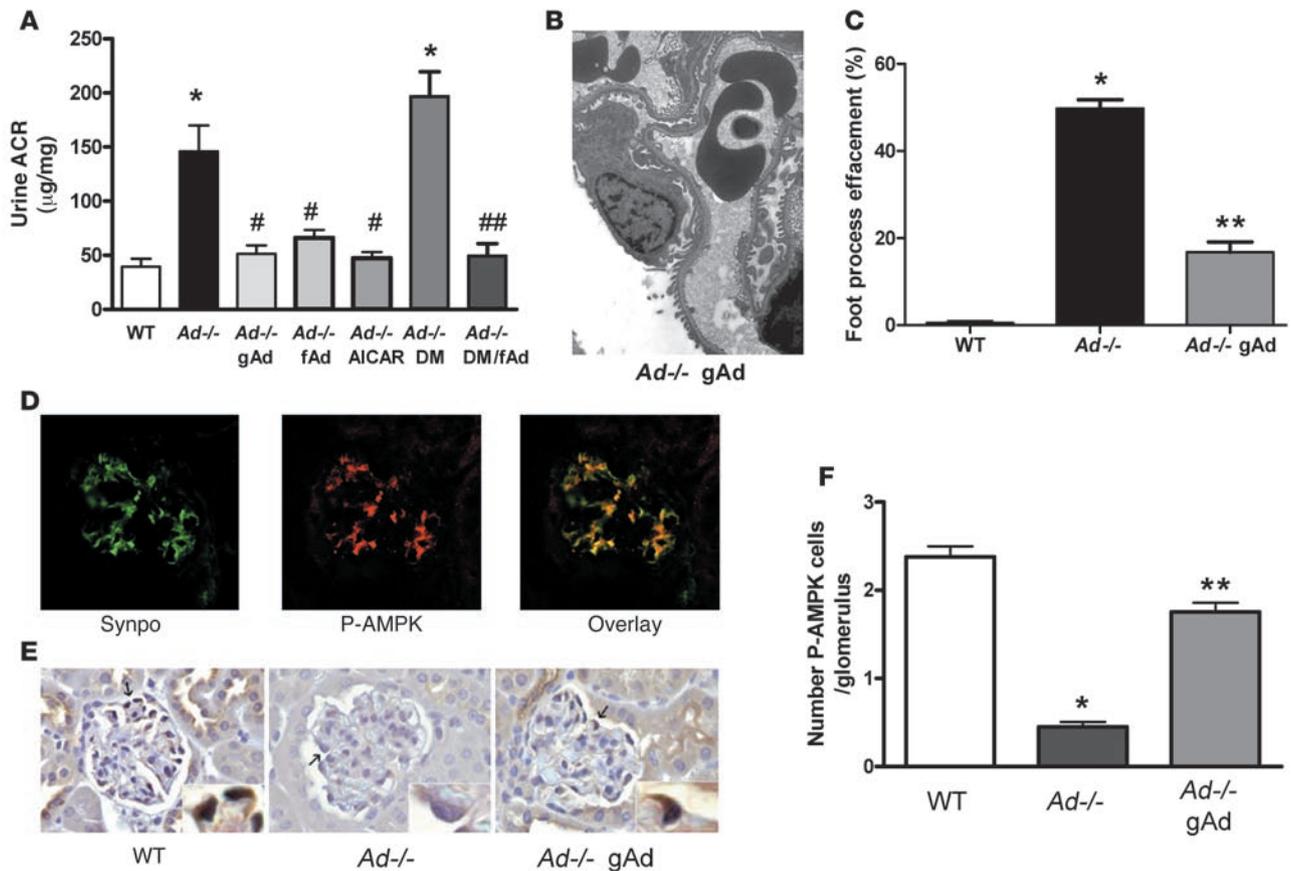
## Methods

**Human subjects.** Subjects were recruited from an AA cohort that has been examined prospectively since late adolescence (58). Inclusion criteria were BMI greater than 30 and no history of known diabetes or kidney disease (serum creatinine,  $\leq 1.4$  mg/dl). Written informed consent was obtained from all subjects. Measurements included height; weight; calculated BMI; blood pressure; 3 separate urine ACRs assayed from timed overnight collections; and levels of adipocytokines, adiponectin, IL-6, and PAI-1. Insulin sensitivity was measured via an insulin clamp procedure as previously described (59). Urine albumin was measured by RIA (Diagnostic Products Corp.). Plasma adiponectin was measured with an RIA kit (Linco Inc.). Plasma IL-6 and PAI-1 were measured by ELISA (R&D Systems). The protocol was approved by the Thomas Jefferson University Institutional Review Board.

**Animals.** Male  $Ad^{-/-}$  mice on the C57BL/6 genetic background, as previously described (18, 31), were chosen for this study. The genotype of each mouse was determined by PCR analysis of mouse tail DNA as

previously described (31). All animal procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. Mice were given standard rodent chow (Purina 5010) and water ad libitum. Urine was collected in Nalgene metabolic cages at various time points. A cohort of male WT and  $Ad^{-/-}$  mice at 2 months of age were made diabetic with a multiple low-dose streptozotocin protocol as previously described (60). Blood glucose was measured with Accucheck (Roche). Urine in diabetic and nondiabetic mice was collected at baseline, 2 months after diabetes induction, and 4 months after diabetes induction (2, 4, and 6 months of age, respectively).

**Interventional animal studies.** The mice were treated with recombinant human gAd (PepROTech) administered 25  $\mu$ g/mouse i.p. twice a day for 10 consecutive days, or fAd administered using an AAV vector (AAV2/8 CMV fAd virus,  $4 \times 10^{11}$  GC/mouse i.p. for 10 days). A separate group of  $Ad^{-/-}$  mice was treated with AICAR with a single i.p. dose of 300 mg/kg. The control animals were given PBS alone or control AAV. Twenty-four hour urines from each mouse were collected before treatment and on the last day of the treatment period. The urine albumin and creatinine were measured with a mouse Albuwell ELISA kit and a Creatinine Companion kit (Exocell Inc.; refs. 60, 61). As an index of oxidant stress, timed urine collections were also analyzed for hydrogen peroxide by Amplex red assay (Invitrogen) following the manufacturer's protocol. Portions of liver, muscle, and kidney were snap-frozen in liquid nitrogen for RNA and protein isolation. An additional aliquot of normal kidney was frozen in OCT for immunofluorescent staining. Portions of kidney cortex were fixed in buffered formalin and embedded in paraffin, and a separate aliquot of kidney cortical tissue was cut into 1-mm<sup>3</sup> pieces and fixed in 2.5% glutaraldehyde in Millonig solution and embedded in PolyBed 812 (Polysciences Inc.) for EM analysis. Individual capillary loops were evaluated and quantitated for the degree of foot process effacement by a single investigator following a previously published method (62).



**Figure 6**

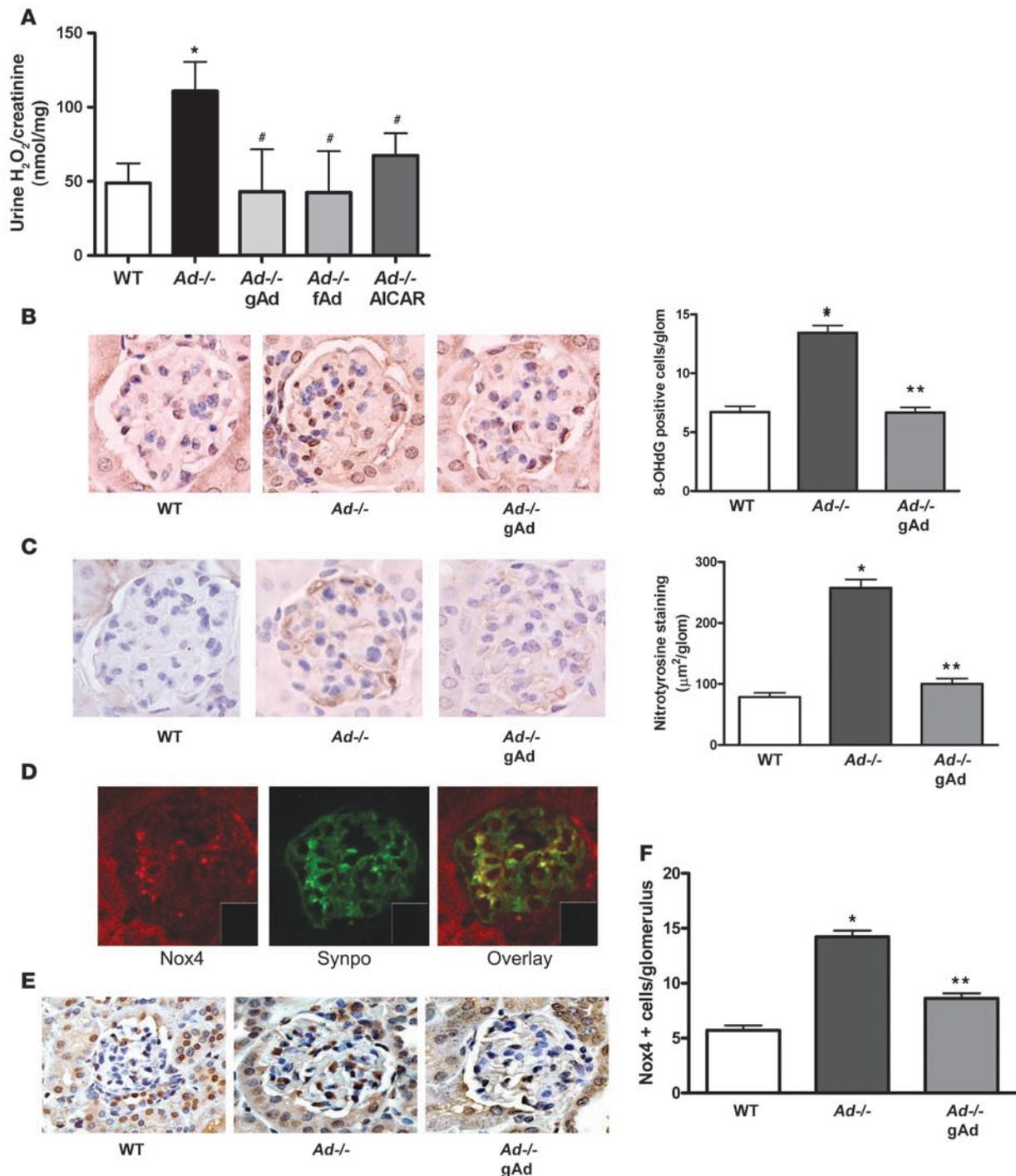
Adiponectin restores normoalbuminuria and increases AMPK activity. (A) *Ad<sup>-/-</sup>* mice at 4 months of age were treated with saline, gAd, fAd, or AICAR, and urine ACR was measured. In addition, *Ad<sup>-/-</sup>* diabetic mice (2 months of diabetes) were treated with fAd, gAd, fAd, and AICAR treatment significantly decreased the urine ACR to the control values seen in WT mice ( $n = 7-10$  per group). \* $P < 0.05$  versus WT; # $P < 0.05$  versus *Ad<sup>-/-</sup>*; ## $P < 0.05$  versus *Ad<sup>-/-</sup>* DM. (B) Podocyte foot process fusion in *Ad<sup>-/-</sup>* mice was reduced with gAd treatment (compare with Figure 2D). (C) Semiquantitation of the degree of foot process effacement in WT mice, *Ad<sup>-/-</sup>* mice, and *Ad<sup>-/-</sup>* mice treated with gAd. Values represent percent foot process effacement of individual glomeruli. \* $P < 0.05$  versus WT; \*\* $P < 0.05$  versus *Ad<sup>-/-</sup>*. (D) AMPK activity was demonstrated in normal glomerular podocytes by double labeling with P-AMPK antibody and podocyte-specific synaptopodin (Synpo) antibody. (E) AMPK activity was reduced in glomeruli of *Ad<sup>-/-</sup>* mice and increased by adiponectin treatment. Mouse kidneys were immunostained by light microscopy with antibody specific for p-AMPK $\alpha$  as described Methods. Arrows denote p-AMPK $\alpha$ -positive podocytes. Insets show higher magnification of the same cells. Photomicrographs are representative of 50 glomeruli from each mouse kidney ( $n = 4$  per group). (F) Quantitation of p-AMPK $\alpha$ -positive cells per glomerulus ( $n = 4$  per group). \* $P < 0.05$  versus WT; \*\* $P < 0.05$  versus *Ad<sup>-/-</sup>*. Values are mean  $\pm$  SEM. Original magnification,  $\times 5,000$  (B);  $\times 40$  (D and E);  $\times 100$  (E, insets).

**Podocyte cell culture.** Conditionally immortalized mouse podocytes, kindly provided by P. Mundel (Mt. Sinai School of Medicine, New York, New York, USA), were cultured as previously described (63). Differentiated podocytes were cultured at 37°C for 8–10 days without IFN- $\gamma$  in DMEM containing 5.5 mmol/l glucose and 5% FCS.

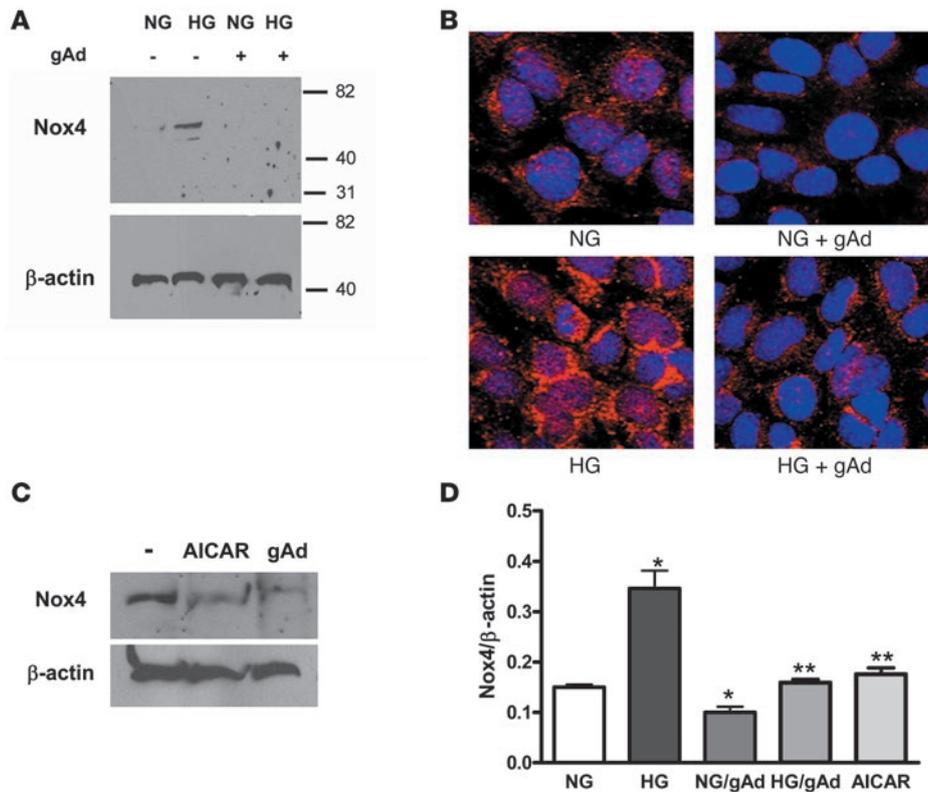
For the permeability assay, a modification of a previously published protocol was adopted (37). Differentiated podocytes ( $0.2 \times 10^6$  podocytes/well) plated on type I collagen-coated 24-well Transwell plates (Corning) were serum-starved overnight when confluent. Cells were then modulated with 3  $\mu\text{g/ml}$  gAd (PepROTech Inc.), 3  $\mu\text{g/ml}$  fAd (PepROTech Inc.), or 1 mM ARA (Sigma-Aldrich) for 24 h or with 1 mM AICAR for 1 h. Cells were then washed twice with PBS supplemented with 1 mM each MgCl<sub>2</sub> and CaCl<sub>2</sub>. The upper compartment was refilled with 0.25 ml RPMI 1640 alone and the lower compartment with 0.5 ml RPMI 1640 supplemented with 40 mg/ml BSA and incubated for 2 hours at 37°C. Total protein concentration in the upper compartment was determined using a Bio-Rad protein assay (Bio-Rad Laboratories).

**Immunoblotting.** Podocytes were cultured as described above, and, after differentiation in 6-well plates, the culture medium was replaced with serum-free DMEM with normal (5.5 mM D-glucose) or high glucose (25 mM D-glucose) for 24 h. In separate wells, podocytes were treated with 3  $\mu\text{g/ml}$  gAd for 6 h or 1 mM AICAR for 1 h prior to modulation with glucose. Immunoblotting was performed as described previously (64). Primary antibodies included rabbit AdipoR2 polyclonal antibody (Alpha Diagnostics), p-AMPK $\alpha$  polyclonal antibody, rabbit anti-AMPK $\alpha$  monoclonal antibody (Cell Signaling Technology), and polyclonal rabbit antibody specific for Nox4, as previously described (65, 66). For verifying equal loading, antibody to  $\beta$ -actin was used.

**Immunohistochemistry.** Immunocytochemistry was performed as described previously (67). Differentiated podocytes seeded on coverslips were serum-starved overnight, and gAd was added. After 24 h adiponectin introduction, cells were fixed in 3.7% paraformaldehyde. Primary antibodies included p-AMPK $\alpha$  (Thr172) rabbit monoclonal antibody

**Figure 7**

Regulation of oxidant stress and Nox4 by adiponectin. **(A)** Urinary levels of hydrogen peroxide were reduced by gAd, fAd, or AICAR treatment in *Ad<sup>-/-</sup>* mice ( $n = 7-10$  per group). \* $P < 0.05$  versus WT; # $P < 0.05$  versus *Ad<sup>-/-</sup>*. **(B)** Glomerular 8-OHdG was increased in *Ad<sup>-/-</sup>* kidneys and reduced with gAd, demonstrated by light microscopy immunostain and quantitation of 8-OHdG-positive cells per glomerulus ( $n = 4$  per group). \* $P < 0.05$  versus WT; \*\* $P < 0.05$  versus *Ad<sup>-/-</sup>*. **(C)** Glomerular nitrotyrosine was increased in *Ad<sup>-/-</sup>* kidneys and reduced with gAd treatment, demonstrated by light microscopy immunostain and quantitation of nitrotyrosine staining per glomerulus ( $n = 4$  per group). \* $P < 0.05$  versus WT; \*\* $P < 0.05$  versus *Ad<sup>-/-</sup>*. **(D)** Nox4 was present in podocytes, as well as other glomerular cells and tubular cells, as demonstrated by double labeling with synaptopodin in WT kidney. Insets show representative background staining without primary antibody. **(E)** Light microscopy immunostain demonstrated that Nox4 protein was increased in glomerular cells of *Ad<sup>-/-</sup>* kidneys and reduced with gAd treatment. Photomicrographs are representative of 50 glomeruli from each mouse kidney ( $n = 4$  per group). **(F)** Quantitation of Nox4-positive cells per glomerulus ( $n = 4$  per group). \* $P < 0.05$  versus WT; \*\* $P < 0.05$  versus *Ad<sup>-/-</sup>*. Values are mean  $\pm$  SEM. Original magnification,  $\times 40$  (**B-E**);  $\times 10$  (**D**, insets).



**Figure 8**

Podocyte Nox4 is increased by high glucose exposure and reduced by adiponectin or AICAR. (A) Podocytes grown in the presence of 3 μg/ml gAd for 24 h showed suppression of Nox4 with normal or high glucose exposure. Transferred proteins were immunoblotted with antibody to Nox4 and β-actin. (B) Similar studies were performed with podocytes grown on coverslips, demonstrating reduction of Nox4 protein. (C) AMPK activation with AICAR demonstrated Nox4 protein reduction to a degree similar to that shown by gAd in podocytes grown with high glucose exposure. Shown are representative immunoblots and confocal images from 5 separate experiments. (D) Quantitation of Nox4 relative to β-actin from the immunoblots (*n* = 5). Values are mean ± SEM. \**P* < 0.05 versus normal glucose; \*\**P* < 0.05 versus high glucose.

(Cell Signaling Technology), Nox4 rabbit polyclonal antibody (Novus Biologicals), and ZO-1 (Invitrogen). Nuclear stain was performed with Hoechst 33342 (Invitrogen). Fluorescence images were obtained using a confocal laser fluorescence microscope (LSM-510; Carl Zeiss). ZO-1 linear staining was performed using computer-assisted image analysis. Immunostaining of paraffin-embedded mouse kidneys was performed as described previously (68). Primary antibodies included p-AMPKα (Thr172) rabbit monoclonal antibody, Nox4 rabbit polyclonal antibody (Novus Biologicals), anti-8-OHdG monoclonal antibody (Japan Institute for the Control of Aging), and nitrotyrosine monoclonal antibody (Cayman), used at 1:10 dilution per the manufacturers protocols. Quantitation of p-AMPKα, Nox4, and 8-OHdG-positive cells was performed on 20–50 glomeruli from 4 mice per group. Quantification of the nitrotyrosine-positive area in glomeruli was performed by color-subtractive, computer-assisted image analysis, as described previously (69). For localization studies in frozen mouse kidney tissue, immunofluorescence with overlay was performed with the primary antibodies, rabbit monoclonal antibody p-AMPKα (Thr172) and polyclonal rabbit anti-Nox4, and double staining was performed with the podocyte-specific mouse anti-synaptopodin antibody (Biodesign). Images were captured by confocal laser fluorescence microscope.

**RNA isolation and quantitative real-time PCR.** Total RNA was isolated from liver, muscle, kidney, and differentiated podocytes using TRIzol reagent (Invitrogen) as previously described (70). Real-time PCR was performed as

previously described (70). The primers for AdipoR1, AdipoR2, Nox1, Nox2, Nox4, and β-actin are listed in Supplemental Methods.

**Statistics.** Data are summarized as arithmetic means ± SEM or medians. Data that were normally distributed were used in a Pearson correlation analysis. Data that did not meet the criteria of normally distributed data were used in a Spearman rank correlation. A *P* value less than 0.05 was considered significant. All reported *P* values are 2-sided. Analyses were carried out using Graph Pad Prism software version 4.03 and SPSS version 13.0 for the PC. Differences between data groups were evaluated for significance using independent *t* test of data or 1-way ANOVA and Newman-Keuls post-hoc tests.

**Acknowledgments**

These studies were performed with partial support from NIH grants DK 053867 and DK 076133 (to K. Sharma), DK 63018 and DK 71360 (to B. Goldstein), HL 051547 and DK 046107 (to B. Falkner), and HL 51586 (to L. Chan).

Received for publication May 15, 2007, and accepted in revised form February 20, 2008.

Address correspondence to: Kumar Sharma, Translational Research in Kidney Disease, Department of Medicine, University of California, San Diego, 9500 Gilman Drive, MC 0711, La Jolla, California



92093-0711, USA. Phone: (858) 822-0860; Fax: (858) 822-7483; E-mail: KumarSharma@ucsd.edu. Or to: Barry J. Goldstein, Division of Endocrinology, Diabetes, and Metabolic Diseases, Department of Medicine, Jefferson Medical College, Suite 320, 1015 Walnut Street, Philadelphia, Pennsylvania 19107, USA. Phone: (215) 503-1272; Fax: (215) 923-7932; E-mail: Barry.Goldstein@jefferson.edu.

Stephen R. Dunn's present address is: Cancer Genomics Facility, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania, USA.

Portions of this work were presented in abstract form at the American Society of Nephrology meeting in Philadelphia, Pennsylvania, USA, on November 8–13, 2005.

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