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 (Cedarlane) and goat polyclonal anti-mouse nephrin antibody (Santa Cruz). After incubation with biotin-conjugated secondary antibody, as to adiponectin and nephrin 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 $CCr=(Cu/Cp)\times V$, 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, 5×10^8 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 Interstitum in Remnant Kidney

Immunohistochemical analysis showed abundant immunostaining for adiponectin in the glomeruli and interstitum of the remnant kidney of WT mice at 8 weeks after subtotal nephrectomy but not in the control glomeruli and interstitum (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 interstitum 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).

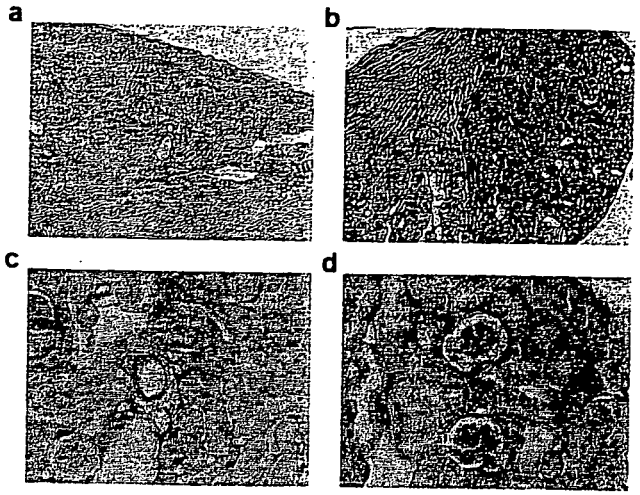

Figure 1. Representative results of immunohistochemical analyses for adiponectin in non-operated WT (a and c) and WT mice after subtotal nephrectomy (b and d). Control nonoperated mice showed limited glomerular and tubulointerstitial staining for adiponectin (a and c), whereas glomerular and tubulointerstitial 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 (mm^2): $2.55\pm0.01\times10^{-3}$ for KO mice (n=6), $2.52\pm0.11\times10^{-3}$ 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: $2.72\pm0.11\times10^{-3}$ and $3.13\pm0.11\times10^{-3}$ mm^2, 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%; $P<0.05$). Nephrin 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 nephrin, quantative real-time PCR was performed in the control and remnant kidney. Subtotal nephrectomy resulted in significant reduction of nephrin mRNA levels in the remnant kidney in KO mice than in WT mice (supplemental Figure I-e). Adiponectin deficiency had no effect on nephrin 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, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$, were increased in the remnant kidney in KO mice than in WT mice (Figure 3d). On the other hand, there were no significant differences in the mRNA expression levels of catalase and Cu,Zn-SOD, as antioxidant enzymes, between KO and WT mice, although subtotal nephrectomy significantly reduced antioxidant enzyme mRNA levels both of 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

	Control		Subtotal Nephrectomy	
Parameters	WT (n=6)	KO (n=6)	WT (n=6)	KO (n=8)
Body Weight, g	32.7±1.2	31.6±1.2	27.6±0.5	27.1±0.7
Systolic blood pressure, mm Hg	103.0±1.7	103.0±1.3	103.2±3.0	102.0±0.8
Heart rate, beat/min	681.2±12.9	675.8±20.2	705.0±24.1	717.4±12.3
No. of glomeruli/section	184.6±10.9	176.5±7.9	73.5±5.7	70.3±2.1
Blood urea nitrogen, mg/dl	29.3±3.6	28.0±1.8	70.0±4.0	72.5±4.5
Serum creatinine, mg/dl	0.11±0.02	0.09±0.01	0.25±0.01	0.25±0.02
Creatinine Clearance, μl/min	208.1±22.0	174.7±35.8	113.0±14.9	101.5±10.9
Serum adiponectin, μg/ml	19.0±1.4	ND	32.2±3.9	ND
Urinary albumin, mg/g Cr	35.2±21.5	27.1±6.1	52.2±11.4	99.4±14.8*

Data are mean±SEM. $P<0.05$ compared with WT after subtotal nephrectomy. ND indicates not detectable.

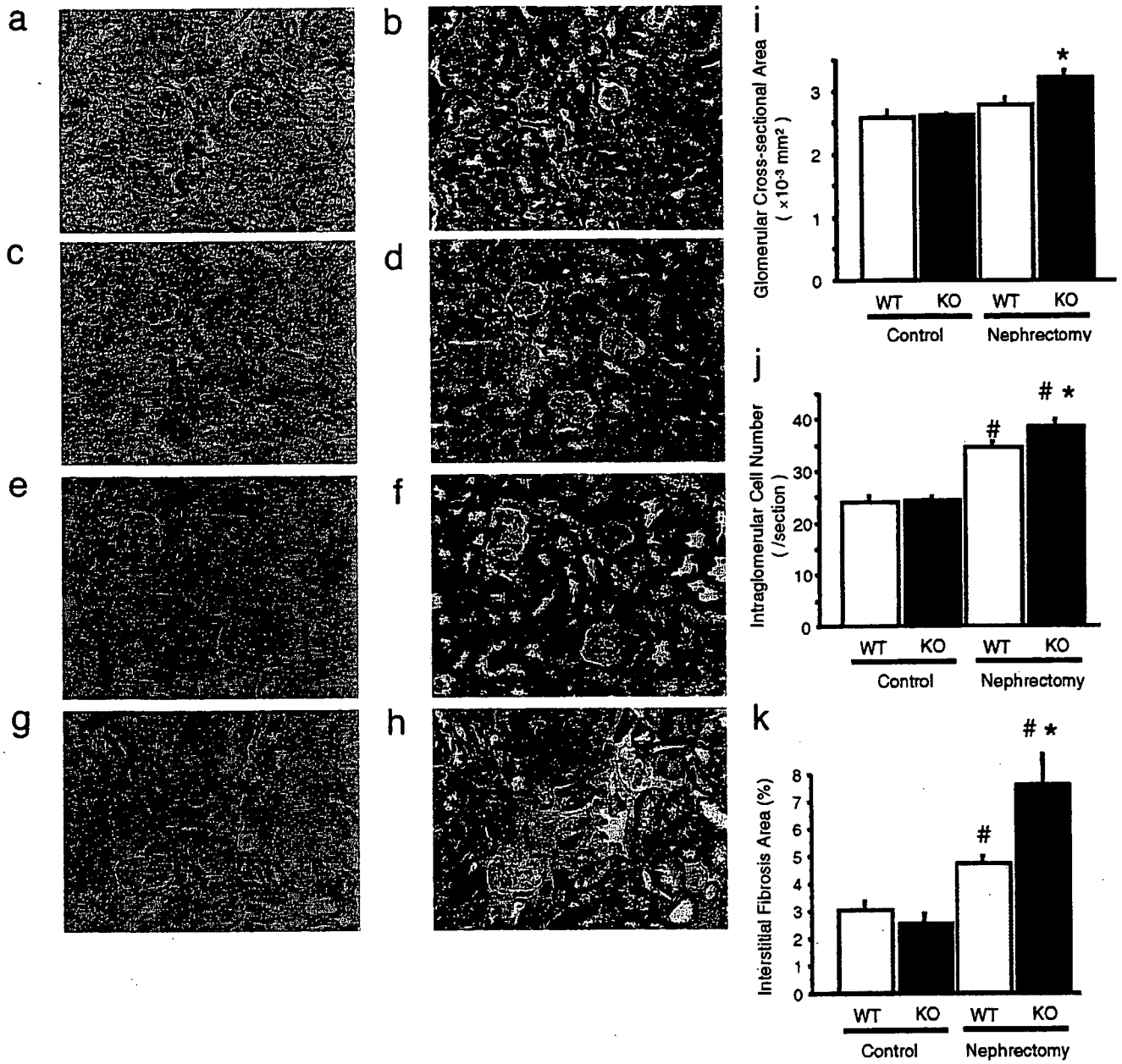

Figure 2. a through h, Histology of the kidneys of nonoperated WT (a and b), nonoperated KO (c and d), and WT (e and f) and KO (g and h) mice after subtotal nephrectomy. Representative periodic acid-Schiff (PAS)-stained sections (a, c, e, and g) and Masson trichrome stained sections (b, d, f, and h). 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.

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 interstitum 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

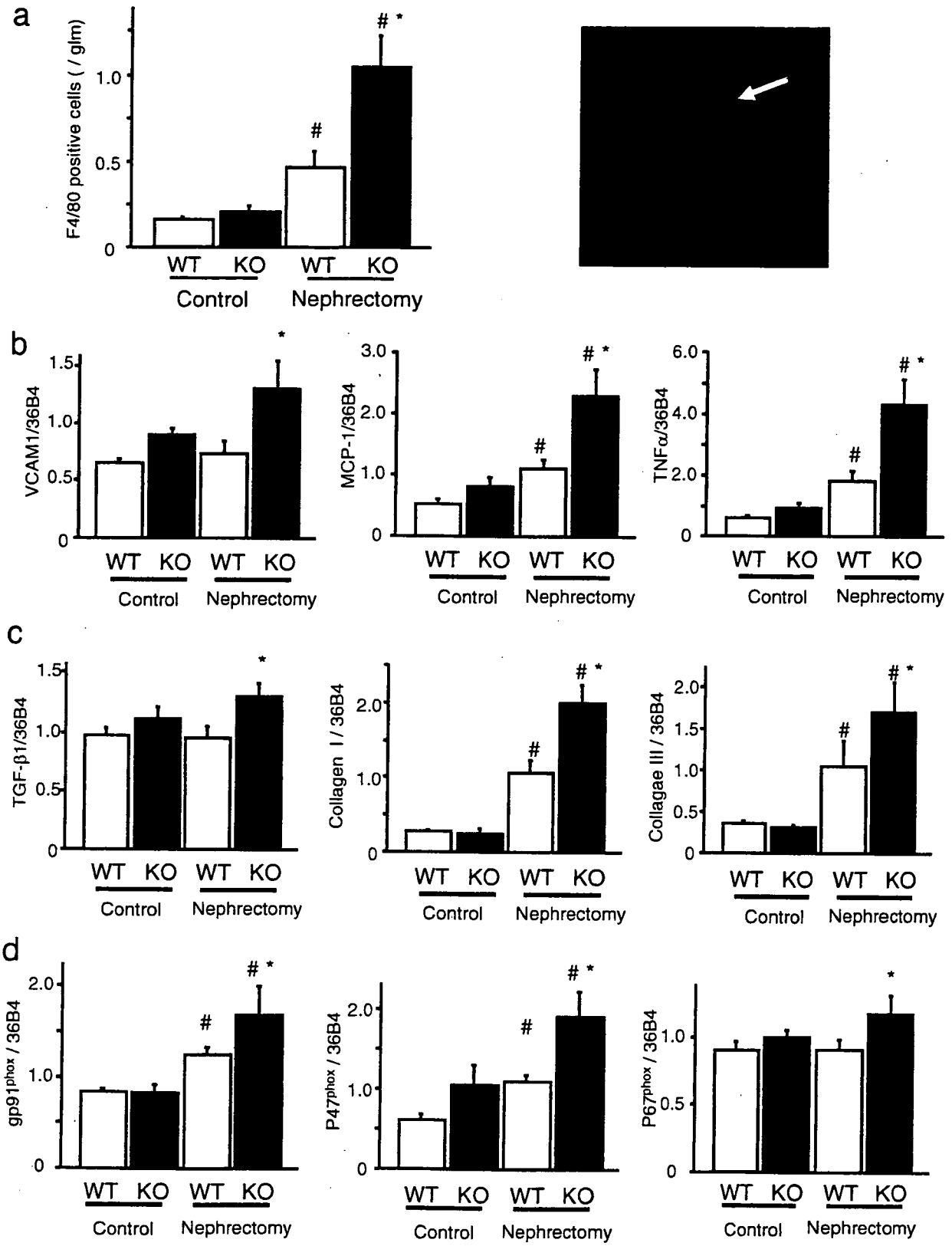

Figure 3. a, Immunofluorescence study for F4/80 in glomeruli of nonoperated WT and KO and renal-ablated WT and KO mice. Right panel shows representative F4/80 staining in a glomerulus. Immunohistochemical analysis revealed increased number of glomerular infiltrating F4/80-positive macrophages in KO mice 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, gp91^{phox}, p47^{phox}, and p67^{phox} 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 urinary 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, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ 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.[1] 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.[19,20] 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.[21] Microalbuminuria, defined as urine albumin to urine creatinine ratio of 30 to <300 μg/mg,[22] is an established risk factor for cardiovascular morbidity and mortality in individuals with hypertension and diabetes mellitus and even in healthy subjects.[23,24] Dysfunction of the vascular endothelium, chronic low-grade inflammation, and oxidative stress are common pathophysiological findings in microalbuminuria and cardiovascular disease.[16,25] 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,[26] oxidative stress, and inflammatory changes comprising cell infiltration or proliferation and accumulation of extracellular

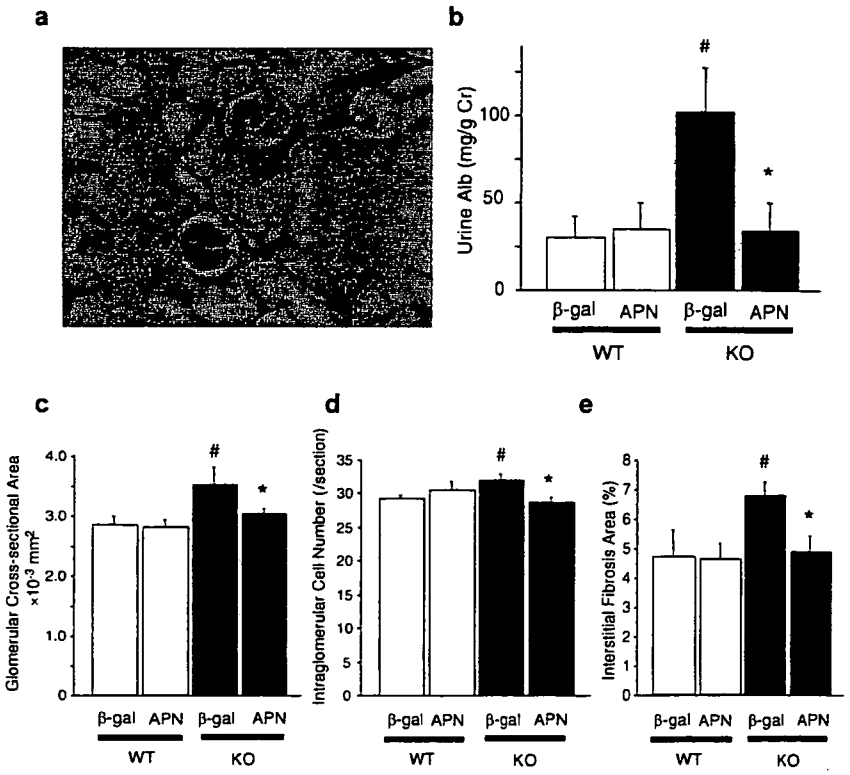

Figure 4. a, Representative results of immunohistochemical analyses for adiponectin in Ad-APN-treated KO mice after subtotal nephrectomy. Accumulation of adiponectin in glomeruli and tubulointerstitium originated from the blood stream. Magnification ×200. b, Urinary excretion of albumin/Cr (mg/g Cr) in KO and WT mice treated with Ad-APN or Ad-β gal. Ad-APN treatment significantly decreased urinary excretion of albumin/Cr (mg/g Cr) in KO than in KO/Ad-β gal after subtotal nephrectomy, whereas no differences were observed between WT/Ad-β gal and WT/Ad-APN after subtotal nephrectomy. The mean area of glomerular cross-section (c), number of intraglomerular cells (d), and area of interstitial fibrosis (e) in KO and WT mice treated with Ad-APN or Ad-β gal are shown. The mean glomerular cross-sectional area, number of intraglomerular cells, and tubulointerstitial fibrotic area were significantly lower in Ad-APN–treated KO mice than Ad-β gal–treated KO mice. #$P<0.05$ for Ad-β gal–treated WT mice. *$P<0.05$ for Ad-β gal–treated KO mice.

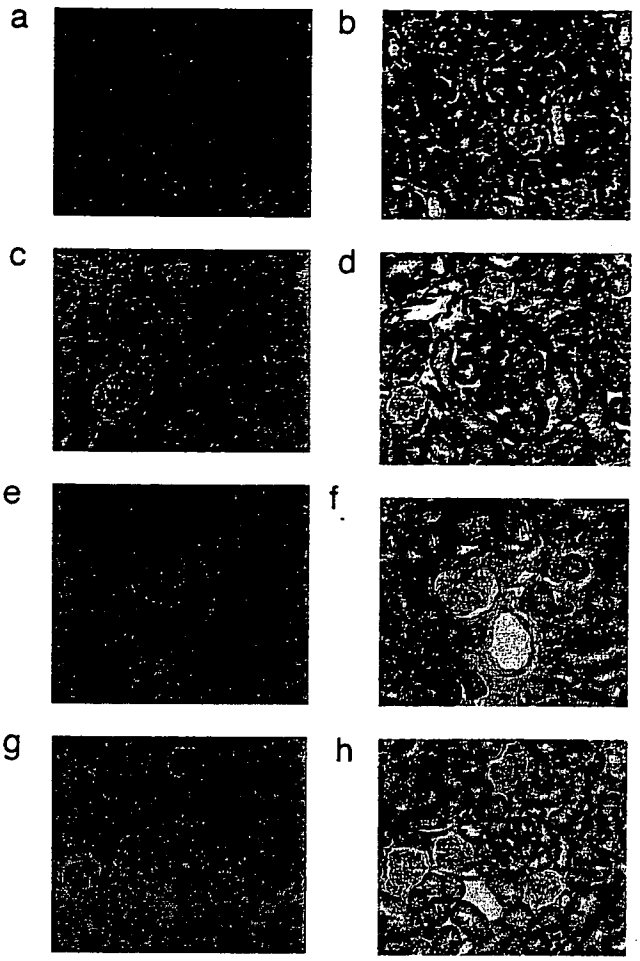

Figure 5. a through h, Histology of kidneys of WT/Ad-β gal (a and b), WT/Ad-APN (c and d), KO/ Ad-β gal (e and f) and KO/Ad-APN (g and h) after renal ablation. Representative periodic acid-Schiff (PAS)-stained sections (a, c, e, and g) and Masson trichrome stained sections (b, d, f, and h).

matrix.[27] Moreover, a causal relationship appears to exist between 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 antiinflammatory and antioxidative properties.[7,30,31] Clinically, hypoadiponectinemia is closely associated with increased levels of inflammatory markers such as C-reactive protein and IL-6.[28] In vitro, recombinant adiponectin suppressed TNF-α–induced expressions 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 hemodynamics 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 exacerbates 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 increased AMP-activated protein kinase, peroxidase 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 phagocytic 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 mediated 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 accumulation 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 proinflammatory mechanism and that adiponectin supplementation might be therapeutically beneficial in renal disorders.

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Disclosures

None.

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Effects of Peroxisome Proliferator-Activated Receptor Ligands, Bezafibrate and Fenofibrate, on Adiponectin Level

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Objective—Adiponectin is adipose-specific secretory protein and acts as anti-diabetic and anti-atherosclerotic molecule. We previously found peroxisome proliferators response element in adiponectin promoter region, suggesting that peroxisome proliferator-activated receptor (PPAR) ligands elevate adiponectin. Fibrates are known to be PPARα ligands and were shown to reduce risks of diabetes and cardiovascular disease. Effect of fibrates on adiponectin has not been clarified, whereas thiazolidinediones enhance adiponectin. Thus, we explored the possibility and mechanism that fibrates enhance adiponectin in humans, mice, and cells.

Methods and Results—Significant increase of serum adiponectin was observed in bezafibrate-treated subjects compared with placebo group in patients enrolled in The Bezafibrate Infarction Prevention study. Higher baseline adiponectin levels were strongly associated with reduced risk of new diabetes. Fibrates, bezafibrate and fenofibrate, significantly elevated adiponectin levels in wild-type mice and 3T3-L1 adipocytes. Such an effect was not observed in PPARα-deficient mice and adipocytes. Fibrates activated adiponectin promoter but failed to enhance its activity when the point mutation occurred in peroxisome proliferators response element site and the endogenous PPARα was knocked down by PPARα-RNAi.

Conclusions—Our results suggest that fibrates enhance adiponectin partly through adipose PPARα and measurement of adiponectin might be a useful tool for searching subjects at high risk for diabetes. (***Arterioscler Thromb Vasc Biol.* 2007;27:635-641.**)

Key Words: adipocyte ■ adiponectin ■ fibrate ■ metabolic syndrome ■ peroxisome proliferator-activated receptor

Fibrates have been used in clinical practice for >4 decades as a class of agents known to decrease triglyceride levels. Fibrates are also known to be peroxisome proliferator-activated receptor (PPAR)α ligands. Several clinical studies of fibrates have been performed in large populations. The Bezafibrate Infarction Prevention study (BIP) suggested that bezafibrate prevented cardiovascular events in the subgroup of coronary artery disease patients with high triglycerides.[1] Moreover, further subanalyses demonstrated that the administrations of bezafibrate significantly reduced new-onset diabetes[2,3] and myocardial infarction in the patients with the metabolic syndrome (MS).[4] The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study showed that fenofibrate significantly reduced nonfatal myocardial infarctions and coronary revascularizations, a secondary endpoint, among patients with type 2 diabetes.[5] These favorable clinical outcomes in fibrate studies might be explained by not only its triglyceride-lowering effect but also its various PPARα-mediated pleiotropic effects.

Adiponectin is an adipose-specific secretory protein and acts as an anti-diabetic and anti-atherosclerotic molecule.[6] Furthermore, a number of clinical trials showed that subjects with high levels of circulating adiponectin tend to be protected against type 2 diabetes and myocardial infarction.[7,8] Thiazolidinediones, PPARγ ligands, are well known to increase adiponectin levels in humans via upregulation of adiponectin at the transcriptional level,[9,10] whereas the effect of PPARα ligands fibrates on adiponectin has not been fully explored.

We performed a subanalysis of the BIP study to investigate the effect of bezafibrate on human adiponectin in serum and the impact of baseline adiponectin levels on new-onset diabetes. We also examined the effect of bezafibrate and fenofibrate on adiponectin by using mice and cultured cells.

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TABLE 1. Baseline Characteristics of the Study Population

Characteristics	Bezafibrate (n=146)	Placebo (n=146)	P
Age, y	59.7±6.6	59.3±6.5	0.6
Body mass index, kg/m^2	27.8±3.5	28.6±3.2	0.04
Men (%)	131 (90)	127 (87)	0.5
Past myocardial infarction (%)	108 (74)	116 (79)	0.3
Angina (%)	87 (60)	82 (57)	0.6
NYHA Class ≥2 (%)	44 (30)	36 (26)	0.4
Hypertension (%)	58 (40)	52 (36)	0.4
Current smokers (%)	17 (12)	24 (16)	0.2
Past smokers (%)	88 (60)	83 (57)	0.6
Systolic blood pressure, mm Hg	141±17	137±16	0.053
Diastolic blood pressure, mm Hg	84.2±8.7	83.0±8.1	0.2
Glucose, mg/dL	99.1±13	97.4±13	0.3
Total cholesterol, mg/dL	215±17	214±18	0.8
HDL-cholesterol, mg/dL	33.0±4.8	32.7±5.0	0.6
LDL-cholesterol, mg/dL	147±18	147±16	0.7
Fibrinogen, mg/dL	359±77	363±73	0.6
Triglycerides, mg/dL	167 (158–175)	162 (154–171)	0.5
CRP, mg/dL	3.47 (2.95–4.07)	3.90 (3.37–4.50)	0.3
Insulin, μU/mL	4.31 (3.68–5.04)	5.25 (4.49–6.14)	0.04
HOMA-IR	1.05 (0.89–1.23)	1.25 (1.06–1.46)	0.1

HDL indicates high-density lipoprotein; HOMA-IR, homeostatic index of insulin resistance; LDL, low-density lipoproteins; NYHA, New York Heart Association classification.

Data are mean±SD, geometric mean (95% confidence interval) or N (%) of patients.

Materials and Methods

Subjects

Metabolic and inflammatory parameters were analyzed from stored frozen serum samples obtained from randomly selected patients with the MS who completed a 2-year of prospective, double-blind, placebo-controlled study period. The major inclusion and exclusion criteria for the BIP study, as well as the ethical guidelines, have been previously reported.[1] The mean follow-up period of BIP was 6.2±0.8 (range, 4.7 to 7.6 years).

Definition of the MS and Current Study Population

We applied the cut points for the MS based on the NCEP ATP-III report, with minor modifications as noted previously.[4] Out of 1111 nondiabetic patients with the MS, we randomly selected 348 (31%). The full clinical data and paired blood samples were available in 292 patients (146 patients in bezafibrate and 146 in the placebo groups), which comprised the current study population. We used as the criterion for new diabetes, the detection of fasting blood glucose of ≥126 mg/dL (7 mmol/L) and/or initiating of any type of pharmacological antidiabetic treatment during follow-up.

Laboratory Methods of Human Studies

Detailed data on laboratory methods were given in previous reports.[1] A central laboratory performed all biochemical determinations. For the purpose of the present study, serum samples, which had been taken at baseline from each study participant and stored at −70°C, were thawed and assayed for adiponectin levels using enzyme-linked immunosorbent assay kits (B-Bridge International, Inc, Sunnyvale, Calif). The inter-assay and intra-assay variability of the adiponectin test in our study was 5.9% and 3.2%, respectively. The homeostatic indexes of insulin resistance were calculated according to the homeostasis model of assessment as follows:

Homeostatic indexes of insulin resistance =fasting insulin (μU/mL) × fasting glucose (mmol/L) / 22.5 (or fasting glucose in mg/dL/405).

3T3-L1 Cell Cultures

3T3-L1 cells were maintained and differentiated as previously described.[9] On day 4, 3T3-L1 adipocytes were treated with the indicated concentrations of either bezafibrate or fenofibric acid dissolved in dimethyl sulfoxide (DMSO) for 24 hours. An aliquot of the media was subjected to measurement of adiponectin by using enzyme-linked immunosorbent assay kit (Otsuka, Tokushima, Japan). We performed adiponectin promoter analysis as previously described.[9,11] PPARα-and RXRα expression vectors were co-transfected in 3T3-L1 preadipocytes, as previously described.[12] The sequences of the sense siRNAs were as follows: PPARα-RNAi; 5′-CCUUUGAUAUGAUACUUUAdTdT-3′, control-RNAi; 5′-UUCUCCGAACGUGUCACGUdTdT-3′.

TABLE 2. Distribution of Cardiovascular Drugs Among the Study Patients

Drugs	Bezafibrate (n=146)	Placebo (n=146)	P
Beta blockers (%)	56 (38)	61 (42)	0.6
Nitrates (%)	75 (51)	77 (53)	0.8
Calcium antagonists (%)	75 (51)	79 (54)	0.6
Diuretics (%)	28 (19)	19 (13)	0.15
Antiplatelets (%)	95 (65)	87 (60)	0.3
ACE-I (%)	23 (16)	12 (8)	0.05

ACE-I indicates angiotensin-converting enzyme inhibitors.

Data are N (%) of patients.

Animal Preparation and Primary Cultures of Stromal Vascular Cells

PPARα knockout (KO) mice were purchased from the Jackson Laboratory (Bar Harbor, Me). At 8 weeks of age, male wild-type (WT) and PPARα KO mice were fed with CRF-1 (Oriental Yeast, Osaka, Japan) containing either 0.3% bezafibrate, 0.1% fenofibrate, or no compound (control group) for 2 weeks. For preparation of stromal vascular cells (SVCs), subcutaneous adipose tissues were isolated from mice, minced into fine pieces in phosphate-buffered saline containing antibiotic antimycotic solution (Sigma-Aldrich Inc, St. Louis, Mo), and incubated in Dulbecco's modified Eagle medium with 1 mg/mL collagenase type II and antibiotic antimycotic solution at 37°C for 30 minutes. Digested adipose tissues were filtered through sterile 250-μm nylon mesh and centrifuged at 600*g* for 5 minutes to separate floating adipocytes from pellet of SVCs. SVCs were washed with growth media (10% fatal calf serum + Dulbecco's modified Eagle medium containing 200 μmol/L of L-ascorbic acid and antibiotic antimycotic solution), centrifuged at 600*g* for 5 minutes, and resuspended twice. SVCs were seeded onto culture dishes with growth media and differentiated by induction media (growth media with 5 μg/mL of insulin, 250 nM of dexamethasone, 500 μmol/L of 1-methyl-3-isobutyl-xanthin, and 5 μmol/L of troglitazone) after growing confluence. On day 2, the media of SVCs were changed to the maintenance media (growth media with 5 μg/mL of insulin). On day 4, SVC adipocytes were treated with the indicated reagents and harvested after 24 hours of treatment. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

Quantification of mRNA Levels

Total RNAs were extracted by using RNA STAT-60 (Tel-Test Inc, Friendswood, Tex). First-strand cDNA was synthesized from 400 ng of total RNA using Thermoscript reverse-transcription polymerase chain reaction system (Invitrogen Corp, Carlsbad, Calif). Real-time polymerase chain reaction amplification was conducted with the ABI PRISM 7900HT Sequence Detection system and SDS Enterprise Database (Applied Biosystems, Foster City, Calif) using SYBR Green polymerase chain reaction Master Mix (Applied Biosystems). The final result for each sample was normalized to the respective cyclophilin value.

Statistical Analysis for Human Studies

Data were analyzed with SAS software, version 8.2 (SAS Institute, Cary, NC). Comparisons of dichotomous variables and normally distributed continuous variables were performed by χ^2 test and Student *t* test, respectively. Geometric means were used for triglycerides, insulin and C-reactive protein to correct for their skewed distribution. Non-normally distributed variables were compared by the nonparametric Mann-Whitney *U* test, and they were log-transformed for further analysis. Spearman rank correlation coefficients for the study population as a whole were computed for the association between adiponectin levels and other clinical variables.

Because of their skewed distribution, adiponectin was presented as median and interquartile range, and 95% confidence interval (CI). For the assessment of differences after 2 years between bezafibrate and placebo group, an analysis-of-covariance with terms for treatment and baseline values was used based on log-transformed data. Absolute changes (μg/mL) and percent-changes of adiponectin from baseline to 2 years were presented as median and interquartile range and compared using Mann-Whitney *U* test. To explore the risk of clinical events associated with reduced adiponectin levels, we evaluated the development of new diabetes in accordance with tertiles of baseline adiponectin level. Linear trend in crude rates of new diabetes onset was assessed by the Mantel-Haenszel χ^2 test. Age and multivariable adjusted hazard of developing diabetes were computed with the Cox proportional hazard model to account for differences in length of follow-up and correlation of covariates. The variables included in the analysis were age, gender, adiponectin tertiles, body mass index, glucose, insulin, C-reactive protein, triglycerides (log-transformed), and the use of angiotensin-converting enzyme inhibitors. Model performance was assessed with C-statistics and the area under the receiving operating curve. $P<0.05$ was considered as statistically significant.

Statistical Analysis for the In Vivo and In Vitro Experiments

Results were expressed as the mean±SEM of *n* separate experiments. Differences between groups were examined for statistical significance using Student *t* test or ANOVA with Fisher protected least significant difference test. $P<0.05$ denoted the presence of a statistically significant difference.

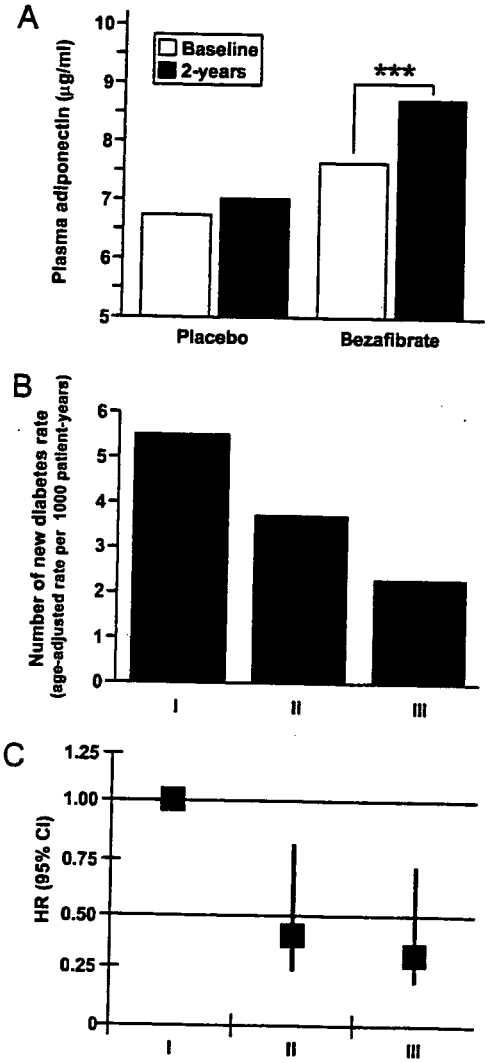

Figure 1. Effect of bezafibrate on serum adiponectin levels and development of diabetes in patients with metabolic syndrome. A, Serum adiponectin levels at baseline and 2 years of follow-up. Data are shown in median values of serum adiponectin. B, Development of new diabetes according to tertiles of baseline adiponectin. Tertiles of baseline adiponectin were as follows: I, adiponectin <5.72 μg/mL (n=96); II, adiponectin 5.72 to 9.25 μg/mL (n=99); III, adiponectin ≥9.26 μg/mL (n=97). Probability value for linear trend in proportions is 0.025. C, Hazard ratio for new diabetes development according to tertiles of baseline plasma adiponectin. Hazard ratio was calculated under adjusting for age, gender, glucose, insulin, C-reactive protein, triglyceride, body mass index, and use of angiotensin-converting enzymeinhibitors. Vertical bars indicate 95% CI. ***$P<0.001$, compared with placebo group.

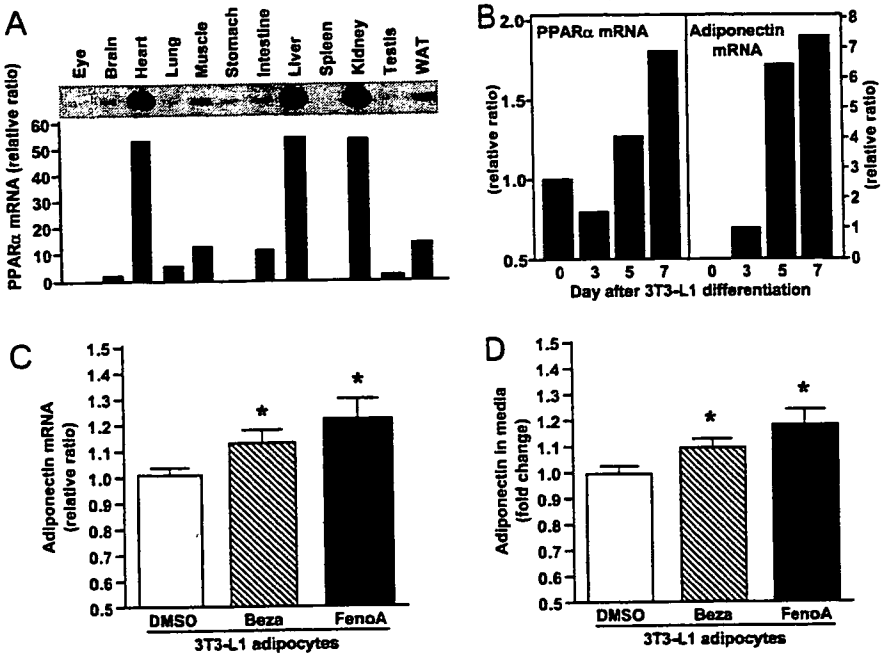

Figure 2. PPARα expressions and effect of fibrates on adiponectin. A, Tissue distribution of mouse PPARα mRNA by northern blotting. Amount of brain PPARα mRNA level was set at 1, and the mRNA levels of PPARα were represented in relative ratio to brain PPARα. B, Gene expressions of PPARα (left panel) and adiponectin (right panel) during differentiation of 3T3-L1 cells. C, Adiponectin mRNA levels in 3T3-L1 adipocytes treated with 10 μmol/L of bezafibrate or fenofibric acid for 24 hours. D, Adiponectin protein levels in cultured media. An aliquot of media from 12 to 24 hours after treatment was subjected to adiponectin measurement. WAT indicates white adipose tissue; Beza, bezafibrate; FenoA, fenofibric acid. C and D, Results are the ratio of the value of nontreated cells (DMSO). Values are mean±SEM; n=6 for each treatment. *$P<0.05$ compared with the values of DMSO.

Results

Baseline Data and Correlations in Subjects

Patients in the placebo and bezafibrate groups were well-balanced in terms of clinical and laboratory baseline characteristics (Table 1). The study groups were similar in regard to age, gender, and the prevalence of the most relevant cardiovascular diseases and risk factors (myocardial infarction in the past, hypertension, heart failure, peripheral vascular disease, anginal syndrome). No significant differences between the groups were found for cholesterol, blood pressure, fasting glucose, triglycerides, fibrinogen, C-reactive protein, and homeostatic indexes of insulin resistance. Among patients on placebo, body mass index and fasting insulin were somewhat higher and systolic blood pressure lower than in patients on placebo. Data regarding treatment with cardiovascular drugs among the study groups are presented in Table 2. Nitrates, calcium antagonists, beta blockers, and antiplatelet drugs (mainly aspirin) were the most commonly used medications. The use of angiotensin-converting enzyme inhibitors was somewhat lower in patients on placebo. There were no significant differences in the proportion of patients receiving the other cardiovascular drugs.

The natural logarithm of adiponectin at baseline was significantly positively correlated with age ($r=0.22$, $P=0.0002$), high-density lipoprotein cholesterol ($r=0.26$, $P=0.0001$) and inversely correlated with natural logarithm of triglycerides ($r=-0.15$, $P=0.009$).

Bezafibrate Treatment Elevates Serum Adiponectin Levels in Human Subjects

No significant differences between the placebo and bezafibrate groups were found for adiponectin levels at baseline (placebo group: median, 6.75 μg/mL; interquartile range, 4.97 to 9.83; bezafibrate group: median, 7.64 μg/mL; interquartile range, 5.20 to 10.6; $P=0.2$). During 2 years of follow-up, there were no significant changes in the adiponectin level of placebo group, whereas the median adiponectin level significantly increased in bezafibrate groups (Figure 1A). Percent-changes in adiponectin level of placebo group were non-significant as well. In contrast, the median percent-changes in bezafibrate group increased significantly by 9.8% (interquartile range, −8.54 to 40.8%; $P<0.0001$). The inter-group differences in percentage changes were in favor of bezafibrate ($P=0.02$). Adiponectin level of bezafibrate group was also higher than that of placebo group after 2-year follow-up (placebo group: median, 7.03 μg/mL; interquartile range, 5.22 to 9.75; bezafibrate group: median, 8.71 μg/mL; interquartile range, 5.53 to 12.10; $P=0.006$).

Development of New Diabetes According to Adiponectin Levels at Baseline

Development of new diabetes was recorded in 69 patients during the mean 6.2-year follow-up period: 33 (11.3%) in bezafibrate and 36 (12.3%) in placebo group ($P=0.4$). A significantly reduced risk for development of new diabetes was demonstrated for the highest tertile of adiponectin (age-adjusted hazard ratio and 95% CI: 0.46 and 0.25 to 0.86). The linear trend across the tertiles was significant and demonstrated a higher risk for development of new diabetes ($P=0.025$) in the lowest tertile of adiponectin (Figure 1B). Multivariable analysis identified adiponectin as an independent predictor of reduced risk of new diabetes development. Adjusting for age and glucose, hazard ratio estimates (95% CI) were 0.51 (0.29 to 0.90) for comparison of II versus I tertile, and 0.44 (0.24 to 0.83) for III versus I tertile (c=0.81). Further adjustment for gender, insulin, C-reactive protein, triglycerides, body mass index, and use of angiotensin-converting enzyme inhibitors resulted in hazard ratio (95% CI) of 0.35 (0.17 to 0.72) and 0.43 (0.23 to 0.81) for tertiles III and II versus I, respectively (Figure 1C) (c=0.77). Another significant variable associated with future overt type 2 diabetes in patients with MS was glucose level (10 mg/dL increment) with hazard ratio 2.50 (95% CI, 1.96 to 3.19).

Fibrates Enhance Adiponectin at the Transcriptional Levels

Bezafibrate is known to be a pan-PPAR agonist that activates not only PPARα but also PPARγ, and fenofibrate is also shown to possess slight PPARγ activation.[13] We previously identified peroxisome proliferators response element (PPRE) site locating on adiponectin gene,[11] and thus we investigated in vitro and in vivo whether fibrates elevate adiponectin through PPARα. Tissue distribution of mouse PPARα showed that PPARα mRNAs were highly expressed in heart, liver, and kidney, whereas its mRNA level of white adipose tissue was similar to skeletal muscle (Figure 2A). In cultured 3T3-L1 cells, PPARα mRNA levels were increased in parallel with the adipocytes differentiation, and adiponectin mRNA was also detected on the third day and increased after differentiation (Figure 2B). We observed the significant increase of adiponectin mRNA in bezafibrate-treated and fenofibrate acid-treated 3T3-L1 adipocytes (Figure 2C). Similar results were obtained for changes of adiponectin protein level in media (Figure 2D).

Next, we measured the influence of fibrates on the activity of adiponectin promoter (Adn-promoter) by using 3T3-L1 adipocytes (Figure 3A and 3B) and preadipocytes (Figure 3C). Both 10 and 100 μmol/L of bezafibrate significantly enhanced Adn-promoter activities (Figure 3A, lane 2 versus 4 and 5). Treatment of 10 μmol/L fenofibrate acid tended to elevate Adn-promoter activity, and 100 μmol/L of fenofibrate acid significantly increased its activity (lane 2 versus 9). Pioglitazone (PGZ), as a positive control, also activated Adn-promoter (lane 2 versus 11) as previously described.[9] However, these ligand-dependent activations of Adn-promoter were totally abolished when the point mutation occurred in PPRE site (lane 5 versus 6, 9 versus 10, and 11 versus 12). To determine the effect of fibrates on Adn-promoter activity via endogenous PPARα, we knocked down PPARα by using RNAi (Figure 3B). Introduction of PPARα-RNAi caused 0.5-fold decrease of PPARα mRNA level compared with control RNAi (data not shown). Knockdown of PPARα significantly reduced fibrates-induced activations of Adn-promoter (lane 2 to 9), whereas such reduction was not observed in PGZ-treated cells (lane 10 versus 11). Figure 3C showed the effect of fibrates on Adn-promoter in 3T3-L1 preadipocytes. Co-expression of PPARα with RXRα significantly increased Adn-promoter activity (Figure 3C, lane 1 versus 2). Addition of bezafibrate and fenofibrate acid augmented this increase, respectively (lane 2 versus 3 or 4), but PGZ treatment failed to enhance such increase (lane 2 versus 5).

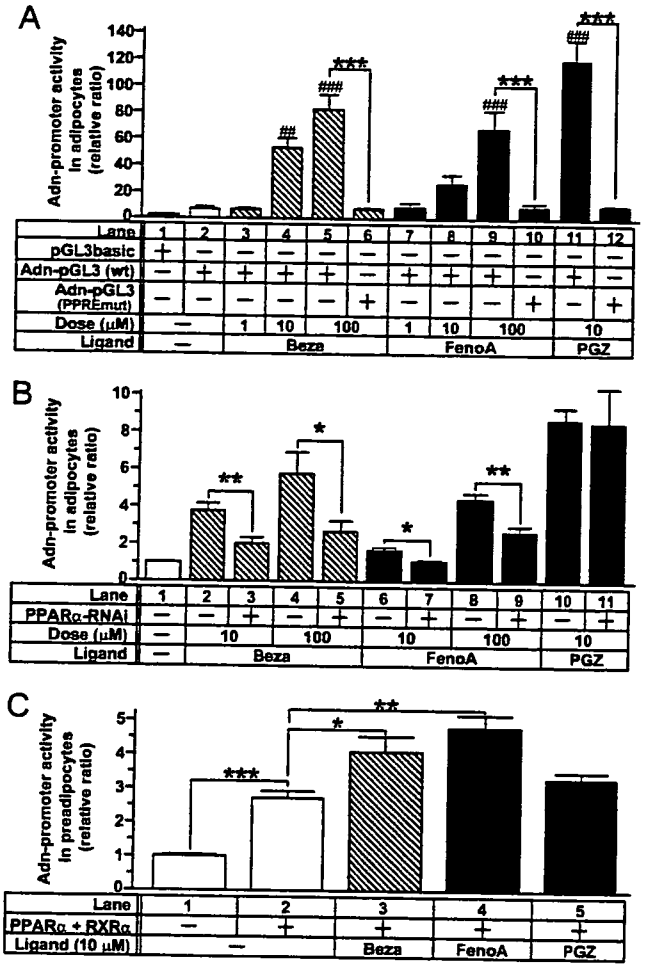

Figure 3. Effect of fibrates on the adiponectin promoter activity. 3T3-L1 adipocytes (A, B) and preadipocytes (C) were transfected with the indicated luciferase reporter constructs. A, Effect of fibrates on adiponectin promoter. B, Effect of fibrates on adiponectin promoter activity in PPARα-knockdown adipocytes. C, PPARα-mediated induction of adiponectin promoter. Adn-pGL3 (wt), pGL3-basic plasmid containing the 5′-flanking region of human adiponectin gene (−908 to +14); Adn-pGL3 (PPREmut), pGL3-basic plasmid containing the point mutation in PPRE site of human adiponectin gene (−908 to +14). Adiponectin-luciferase activity was normalized by pRL-SV40 Renilla luciferase activity and represented in fold-induction from lane 1 of each figure. PGZ indicates pioglitazone. Values are mean±SEM; n=6 for each treatment. ##$P<0.01$; ###$P<0.001$ compared with values of lane 2 (A). *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Effect of Fibrates on Adiponectin in WT and PPARα-Deficient Mice and Cultured Adipocytes

To confirm the PPARα-mediated elevation of adiponectin by fibrates, we conducted the fibrates treatment on WT and PPARα KO mice and cells. We found significant increases of plasma adiponectin levels in both bezafibrate-treated and fenofibrate-treated WT mice at 1 and 2 weeks after administration (Figure 4A). Plasma adiponectin levels of bezafibrate-treated PPARα KO mice increased to a larger extent than the control group at 2 weeks, but its elevation was not statistically significant (KO + control versus KO + bezafibrate; $P=0.0838$). No significant increase of plasma adiponectin level was observed in fenofibrate-treated PPARα KO mice compared with control group (KO + control versus KO + fenofibrate; $P=0.7780$). Adiponectin mRNA levels were significantly elevated in bezafibrate and fenofibrate group compared with control group in WT mice, whereas such elevations were not observed in PPARα KO mice (Figure 4B).

Finally, we tested the elevating effect of fibrates on adiponectin by using SVC adipocytes derived from WT and PPARα KO mice. Bezafibrate and fenofibrate acid significantly increased adiponectin mRNA levels and protein in media in WT mice-derived SVC adipocytes (Figure 4C and 4D, left panel). However, treatments of bezafibrate and fenofibrate acid failed to elevate adiponectin mRNA levels

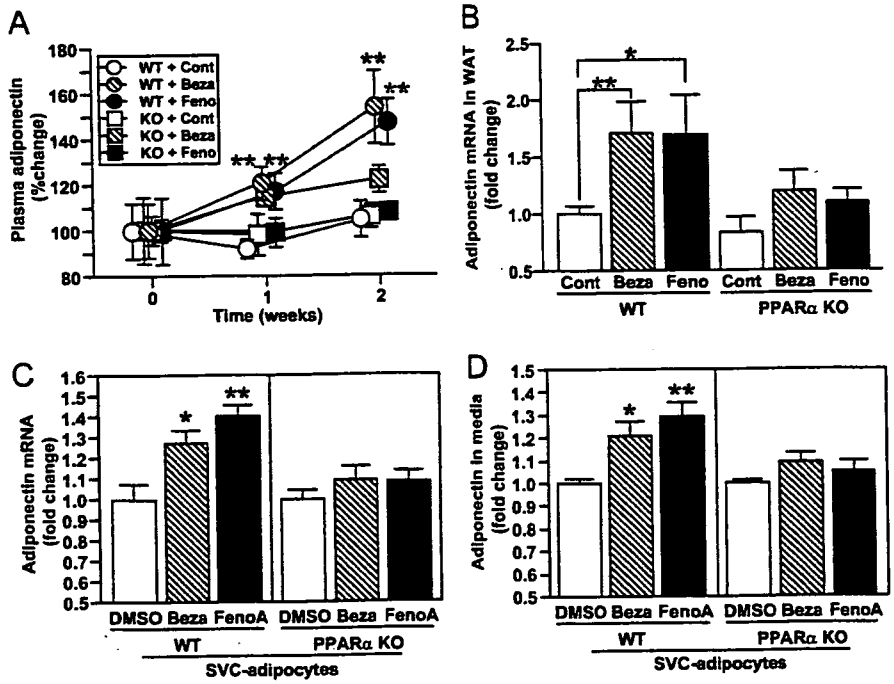

Figure 4. Effect of fibrates on adiponectin in WT and PPARα-deficient mice and cultured cells. A, Time course of plasma adiponectin levels. B, Adiponectin mRNA levels in white adipose tissue (WAT) after 2 weeks of indicated treatments. C, Adiponectin mRNA levels in SVC-adipocytes derived from WT and PPARα KO mice, treated with 10 μmol/L of bezafibrate or fenofibric acid for 24 hours. D, Adiponectin protein levels in cultured media of SVC adipocytes. WT indicates wild-type mice; KO, PPARα knockout mice; Cont, control diet; Beza, bezafibrate; Feno, fenofibrate; FenoA, fenofibric acid. Values are mean ± SEM; n=6 for each treatment. $^{*}P<0.05$; $^{**}P<0.01$, compared with the values of control group (A, B) or nontreated cells (DMSO) (C, D), respectively.

(DMSO versus bezafibrate; $P=0.2536$, DMSO versus fenofibrate acid; $P=0.3166$) and protein (DMSO versus bezafibrate; $P=0.0789$, DMSO versus fenofibrate acid; $P=0.3540$) in PPARα KO mice-derived SVC adipocytes (Figure 4C and 4D, right panel).

Discussion

In this study, we demonstrated: (1) bezafibrate significantly increased serum adiponectin in coronary artery disease patients with the MS; (2) subjects with the highest tertile of baseline adiponectin tended to escape from the development of new-onset diabetes; (3) fibrates enhanced adiponectin via PPRE site locating on its promoter region; and (4) fibrates elevated adiponectin partly through adipose PPARα.

The data regarding fenofibrate were obtained in small populations (n=10 to 20) and shown conflicting results,[14–16] whereas we have found no published data about the effect of bezafibrate on human adiponectin. In animal experiments, one group showed that fenofibrate increased adiponectin,[17] whereas another group observed no change of adiponectin in fenofibrate-treated obese rats.[18] Bezafibrate-administered obese rats showed an elevation of adiponectin levels.[19] In addition, there is no study investigating the effect of fibrates on adiponectin in cultured adipocytes.

PPARα is abundantly expressed in liver, and investigations of PPARα-null mice and fibrate treatments have indicated that PPARα plays an important role in fatty acid oxidation.[20] Fibrates are allowed to exhibit fatty acid oxidation in muscle as well as liver, but the effect of fibrates on adipocytes has not been noted because adipocytes express a small amount of PPARα. The current study showed that PPARα is expressed in adipose tissues as well as in muscle, and this result indicates that adipose tissue also might be a target organ of fibrates. As shown in Figures 3 and 4, fibrates directly and transcriptionally increased adiponectin via adipose PPARα. Consistent with our results, another group recently demonstrated that PPARα mRNA was expressed in adipose tissues and 3T3-L1 adipocytes,[21] and others have shown the existence of PPARα protein in human isolated adipocytes.[22] In addition, PPARα ligands directly stimulated lipolysis in WT adipocytes, but such effect was not observed in PPARα-deficient adipocytes.[23] These previous and present results indicate that PPARα functionally works in adipocytes. Bezafibrate tended to elevate adiponectin in both PPARα-deficient mice and SVC adipocytes (Figure 4A and 4D), which results might be accounted for the partial PPARγ activation induced by bezafibrate known to be a pan-PPAR ligand.[13]

Several clinical studies of PPAR ligands have been conducted in large populations. For example, the PROactive study demonstrated that PGZ improved cardiovascular outcome at main secondary endpoint in patients with type 2 diabetes and also reduced the need to add insulin therapy to glucose-lowering regimens compared with placebo.[24] The BIP and FIELD studies also showed that fibrate treatments achieved beneficial outcomes in selected high-risk groups.[2–6,25] These clinical results of PPAR ligands theoretically might be partly explained by the PPAR ligand-dependent induction of adiponectin as one of PPAR-mediated pleiotropic effects.

Recently, subanalysis of BIP study demonstrated that high levels of adiponectin at baseline were associated with low risk of subsequent diabetes in coronary artery disease subjects with impaired fasting glucose.[26] Here we also showed that high baseline concentration of adiponectin was associated with lower risk for new-onset diabetes in patients with the MS. It should be noted that the randomly selected patients in the placebo and bezafibrate groups in our study present some minor differences regarding degree of obesity, insulin concentrations and use of angiotensin-converting enzyme inhibitors. All these potential confounders were included in the multivariable analysis and did not affect the results. More

randomized control trials and larger longitudinal cohort studies to explore the predictive value of adiponectin measurements in subjects at high risk for diabetes and coronary artery disease are warranted.

In conclusion, fibrates enhance adiponectin partly through adipose PPARα and measurement of adiponectin might be a useful tool for searching for subjects at high risk for diabetes.

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Disclosures

None.

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Reduction of Visceral Fat Is Associated With Decrease in the Number of Metabolic Risk Factors in Japanese Men

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Visceral fat accumulation is associated with the development of metabolic disorders such as glucose intolerance, dyslipidemia, hypertension, and atherosclerotic cardiovascular diseases (1–8). However, the relationship between reduction of visceral fat and decrease in the number of metabolic risk factors has not been defined in the general population. Recently, we developed a new technique, the abdominal bioelectrical impedance analysis (BIA), to evaluate visceral fat area (VFA) (9). The aim of this study was to investigate whether reduction of visceral fat, estimated by the BIA, is associated with a decrease in the number of metabolic risk factors.

RESEARCH DESIGN AND METHODS

The study group comprised 2,336 Japanese men (aged mean ± SD 48.0 ± 10.5 years, BMI 24.2 ± 2.9 kg/m^2), who were employees of Amagasaki City Office, an urban area, and had undergone annual health check-ups in both 2004 and 2005. After the health check-up, the medical staff provided risk factor–oriented, rather than obesity-oriented, health promotion programs to select individuals with visceral fat accumulation and multiple risk factors, with the aim of encouraging a scientific understanding of the spectrum of metabolic syndrome from visceral fat accumulation to atherosclerotic cardiovascular diseases. In this study, we used VFA estimated by the BIA, which was shown to correlate significantly with VFA determined by computed tomography (9). The measurement of VFA by BIA complied with the Guidelines of the Ethical Committees of Osaka University. Informed consent was obtained from all subjects.

Overall obesity was defined as BMI of ≥25 kg/m^2 (10). We investigated the presence of three metabolic risk factors: elevated blood pressure (systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg), dyslipidemia, and dysglycemia/impaired glucose tolerance. Dyslipidemia represented hypertriglyceridemia (fasting or postprandial triglyceride of ≥1.69 or 2.27 mmol/l [11,12], respectively, and/or low HDL cholesterol [HDL cholesterol <1.04 mmol/l]). Dysglycemia/impaired glucose tolerance represented hyperglycemia (fasting or postprandial serum glucose concentration of ≥6.1 or ≥7.77 mmol/l [13], respectively). Subjects who received specific treatment(s) for each of the metabolic risk factors were considered positive for that factor.

Statistical analysis

Fischer's protected least significant difference test and Kruskal-Wallis were used to analyze the relationship between the number of metabolic risk factors and body fat distribution and between change in the number of metabolic risk factors and change in VFA, respectively. Significance level was set at $P < 0.05$.

RESULTS

BMI and VFA varied considerably among individuals. We divided subjects into two groups according to BMI and into two groups according to VFA (Fig. 1*A*). Visceral fat accumulation was defined as VFA of ≥100 cm^2 (10,14). Among 1,497 nonobese subjects (BMI <25 kg/m^2), 401 (26.8%) had visceral fat accumulation. The mean number of metabolic risk factors in subjects with VFA ≥100 cm^2 was significantly higher than in those with VFA <100 cm^2, irrespective of BMI. Importantly, the mean number of metabolic risks was significantly higher in subjects with VFA ≥100 cm^2 plus BMI <25 kg/m^2 than in those with VFA <100 cm^2 plus BMI ≥25 kg/m^2 ($P < 0.0001$) (Fig. 1*A*). These results suggest that assessment of visceral fat accumulation is important in identifying subjects with multiple risk factors.

Next, we investigated the correlation between a 1-year change in VFA (ΔVFA) and change in the number of metabolic risk factors (Δ*n*) within the same period in the 2,336 subjects. VFA decreased within 1 year in 53.1% (1,241 of 2,336) of participants, increased in 33.2% (776 of 2,336), and did not change in 13.7% (319 of 2,336).

We divided these subjects into six bins of ΔVFA (every 15 cm^2). ΔVFA correlated significantly with Δ*n* ($P < 0.0001$) (Fig. 1*B*). When the subjects who received new treatment after 2004 were excluded from the analysis, reduction of visceral fat was also associated with a significant decrease in the number of metabolic risk factors ($P < 0.0001$) (data not shown).

CONCLUSIONS

We demonstrated that *1*) irrespective of BMI (< or ≥25 kg/m^2), subjects with visceral fat accumulation estimated by BIA had a cluster of metabolic risk factors and 2) falls in VFA within 1 year were associated with a significant decrease in the number of metabolic risk factors.

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Abbreviations: BIA, bioelectrical impedance analysis; VFA, visceral fat area.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

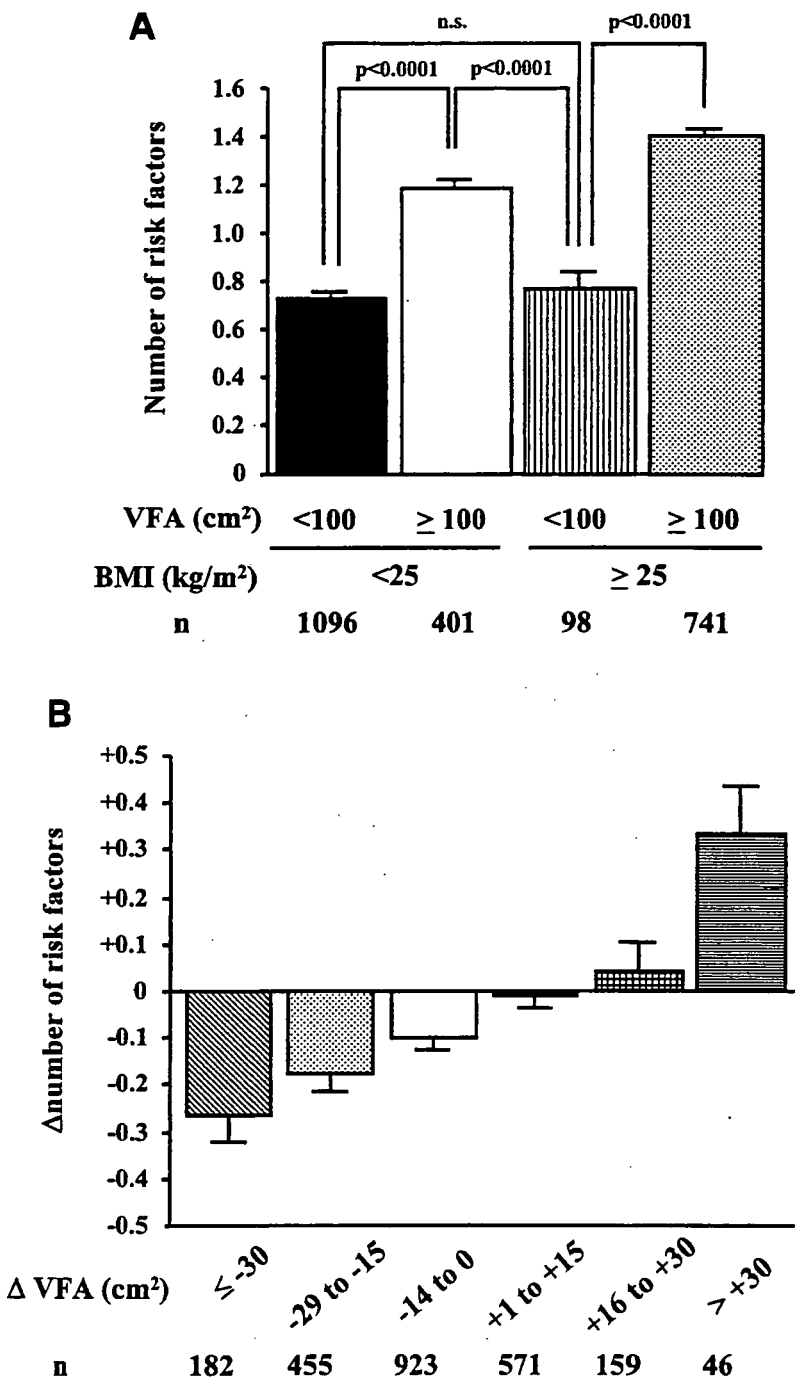

Figure 1—A: *Relationship between number of metabolic risk factors and body fat distribution. Subjects were divided according to their BMI (cutoff value 25 kg/m^2) and VFA (cutoff value 100 cm^2), measured in 2004. Data are means ± SE.* B: *Correlation between changes in VFA and changes in the number of metabolic risk factors. Δnumber of metabolic risk factors represents changes in the number of metabolic risk factors from 2004 to 2005. ΔVFA indicates change in VFA from 2004 to 2005. Subjects were divided into six 15-cm^2 bins of ΔVFA. Data are means ± SE.*

Importantly, our results also demonstrated that subjects with visceral fat accumulation but without overall obesity (VFA ≥100 cm^2 plus BMI <25 kg/m^2) exhibited significantly more metabolic risk factors than overall obese subjects without visceral fat accumulation (VFA <100 cm^2 plus BMI ≥25 kg/m^2). There is ample evidence for the role of visceral fat accumulation in the development of metabolic disorders (4–8,15). Collectively, the above results indicate that assessment of visceral fat accumulation using VFA estimated by BIA is useful for identifying high-risk groups for atherosclerotic cardiovascular diseases.

Our results also demonstrated in a large population sample that changes in VFA within 1 year correlated significantly with Δ*n*. Several reports demonstrated in obese subjects that reduction of visceral fat correlated with improvement in glucose and lipid metabolism (16–19). However, there is little information on the effect of reduction of visceral fat on the number of metabolic risk factors in a large general population sample. Here, we showed in 2,336 subjects that changes in VFA within 1 year correlated significantly with changes in the number of metabolic risk factors. These results suggest that intervention strategies directed toward reduction of visceral fat could result in the reduction or disappearance of risks for atherosclerotic cardiovascular diseases. Since BIA is quite simple and noninvasive for evaluation of visceral fat amount, it could be used in routine clinical practice and large-scale studies for assessment of visceral fat accumulation.

In conclusion, we demonstrated that reduction of visceral fat was closely associated with a decrease in the number of metabolic risk factors in Japanese men.

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Future adverse cardiac events can be predicted by persistently low plasma adiponectin concentrations in men and marked reductions of adiponectin in women after acute myocardial infarction

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Abstract

There is conflicting information about whether mortality after AMI is higher in women than men. We investigated the significance of plasma adiponectin concentrations on major adverse cardiac events (MACE) after acute myocardial infarction (AMI) to delineate any differences between men and women. The study patients consisted of 114 men and 42 women with AMI. The incidence of MACE was significantly higher in women than men during the entire follow-up period ($p < 0.05$). Compared with men for post-AMI MACE, the hazard ratio for women was 5.6 after adjustment for prognostic factors. Killip class ($p < 0.001$) and sex differences ($p < 0.05$) were independent predictors of MACE at 1 year post-AMI. Plasma adiponectin levels in women were significantly higher than men on admission (8.66 μg/mL [range: 6.6–14.08] versus 4.71 μg/mL [range: 3.47–7.27], $p < 0.0001$) and during the post-AMI course (all $p < 0.0001$). Multivariate analysis identified plasma adiponectin level on admission as an independent predictor of MACE in men ($p < 0.001$) and the difference between plasma adiponectin levels at discharge and on admission in women ($p < 0.05$). Patterns of serial changes in plasma adiponectin concentrations are different between men and women and plasma adiponectin concentrations can be used to predict future adverse cardiac events in AMI patients.

Keywords: Myocardial infarction; Adiponectin; Prognosis; Sex

1. Introduction

The issue of whether women have more unfavorable prognosis than men after acute myocardial infarction (AMI) has provoked much controversy [1–4]. Women are reported to have higher relative risk than men in the early phase, especially within 1 year after AMI, and it may be accounted for the older age and more unfavorable risk characteristics of women [1,2]. However, other studies reported no significant differences related to mortality between men and women even after adjusting for age, coronary risk and other prognostic factors [3,4].

Low levels of plasma adiponectin, a representative new member of adipocyte-derived proteins, have been observed in patients with coronary artery disease [5,6]. We previously reported that coronary plaque rupture resulting in the onset

Abbreviations: AMI, acute myocardial infarction; CK, creatinine phosphokinase; ELISA, enzyme-linked immunosorbent assay; HDL, high-density lipoprotein; LAD, left artery descending; LCx, left circumflex artery; MACE, major adverse cardiac events; POBA, plain old balloon angioplasty; RCA, right coronary artery; ROC, receiver operating characteristic; TIMI, thrombolysis in myocardial infarction; VD, vessel disease

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of AMI might reduce plasma concentrations of adiponectin [7]. Adiponectin accumulates in the vascular subendothelial space after damage of the endothelial barrier *in vivo* [8], suggesting that the protein has vessel repair properties. Plasma adiponectin levels may also predict adverse cardiac events and adiponectin may act as a protective factor for the cardiovascular system [9]. High concentrations of adiponectin are associated with future lower risk of AMI [10]. On the other hand, plasma adiponectin levels are lower in men than in women probably due to a selective reduction by testosterone through inhibition of adiponectin secretion from adipocytes [11,12]. However, sex-based differences in clinical outcomes after AMI with regard to adiponectin have not been well defined.

The purpose of the present study was to investigate the serial changes in plasma adiponectin concentrations after AMI in men and women, to delineate any differences between the two sexes with regard to the incidence of adverse cardiac events after AMI, and the role of plasma adiponectin level in such difference.

2. Methods

2.1. Patients

The study patients consisted of 114 men and 42 women who were admitted to our hospital with AMI from October 2000 to March 2004. AMI was defined as elevated myocardial enzyme concentrations, with either typical chest pain persisting longer than 30 min or electrocardiographic changes (including ischemic ST-segment depression, ST-segment elevation, or pathologic Q waves). Elevated enzyme concentrations were defined as peak creatine phosphokinase (CK) concentrations of more than twice the normal upper limit. All patients also met the following criteria: (1) they were admitted to the hospital within 24 h after the onset of AMI, (2) plasma adiponectin concentrations were measured repeatedly up to discharge (mean period of hospitalization of 25 ± 9 days, range: 12–46 days) after the onset of AMI, (3) provided consent to the study, and (4) no post-AMI major adverse cardiac events (MACE) during hospitalization. MACE was defined as the development of the following complications: cardiac-related death, recurrent myocardial infarction, unstable angina, and heart failure requiring emergency rehospitalization. Heart failure defined as dyspnea and/or edema was accompanied by pulmonary congestion on the chest roentgenogram and left ventricular dysfunction on echocardiogram. Patients who were treated with antihypertensive drugs or those whose baseline blood pressure was $\geq$140/90 mm Hg were considered hypertensive. Diabetes mellitus was diagnosed according to the criteria of the World Health Organization [13], however none of the patients was taking any type of thiazolidinedione. Cigarette smoking was defined as active smoking. Killip classes on hospital admission, depending on the clinical manifestations of cardiac failure, were also assessed (Killip 1, no heart failure; Killip 2, S_3 and/or basal lung crepitations; Killip 3, acute pulmonary edema; Killip 4, cardiac shock) [14]. The study protocol was approved by the Human Ethics Review Committee of Kumamoto University and a signed consent form was obtained from each subject.

2.2. Blood sampling, quantification of plasma adiponectin and emergency coronary angiography

In patients with confirmed AMI, blood samples were obtained immediately after hospitalization for measurement of plasma adiponectin concentrations. Furthermore, blood samples were taken every 4 h over the first 24 h for determination of peak CK levels. Venous blood samples were also taken at 24, 72 h, and 7 days after admission and at discharge to measure plasma adiponectin levels. Blood samples for biochemical assessments, such as total cholesterol, triglyceride and high-density lipoprotein (HDL) cholesterol, were obtained after a 12 h fast after admission. Plasma adiponectin levels were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [15].

Emergency coronary angiography was performed in all patients and the allocation of reperfusion therapy was determined by the attending physician and interventional cardiologists independent of this study. The perfusion grade of the infarct-related artery was assessed in accordance with the thrombolysis in myocardial infarction (TIMI) study classification [16]. The final TIMI flow grade was assessed on the final shot of the emergency coronary angiography.

2.3. Follow-up study

After hospital discharge, 156 patients were prospectively followed-up every month with a clinic visit or until occurrence of one MACE. Only the first cardiac event after the enrollment into the study was considered the endpoint in the follow-up analysis. Follow-up data were available for 100% of patients at the entire follow-up period.

2.4. Statistical analysis

Group data of normally distributed continuous variables were expressed as mean $\pm$ S.D., and continuous variables that did not show normal distribution were expressed as the median value (25–75th percentile range). Comparisons of continuous variables were performed using the unpaired *t*-test and the Mann–Whitney *U*-test, as appropriate. Categorical variables were presented by frequency counts, and intergroup comparisons were analyzed by the χ^2-test. We plotted cumulative event curves using the Kaplan–Meier survival method and tested differences between the curves of the two groups for statistical significance by the log-rank analysis. The Spearman two-way test was used to assess the relation between two quantitative variables with non-normal distribution. Variables with non-normal distribution

Table 1
Clinical characteristic of the two study groups

	Men ($n=114$)	Women ($n=42$)	p
Age (years)	61 ± 12	72 ± 10	<0.0001
Time to admission to hospital (h)[a]	2.5 (1.5–4.0)	5.5 (2.0–12.0)	0.0031
Hypertension, n (%)	57 (50)	27 (64)	0.1124
Diabetes mellitus, n (%)	35 (31)	13 (31)	0.9760
Total cholesterol (mg/dL)	206 ± 61	218 ± 47	0.2584
Triglyceride (mg/dL)[a]	131 (91–178)	120 (88–149)	0.4403
HDL cholesterol (mg/dL)	46 ± 14	55 ± 17	0.0011
Smoking, n (%)	81 (71)	7 (17)	<0.0001
Body mass index (kg/m^2)	24 ± 3	22 ± 3	0.0018
Killip class 1/2/3/4, n (%)	96 (84)/15 (13)/2 (2)/1 (1)	32 (78)/6 (15)/1 (2)/2 (5)	0.4311
Culprit artery RCA/LAD/LCx, n (%)	41 (36)/54 (47)/19 (17)	17 (40)/21 (50)/4 (10)	0.5288
Vessel involvement 1VD/2VD/3VD, n (%)	64 (56)/37 (32)/13 (11)	22 (53)/14 (33)/6 (14)	0.8628
Reperfusion therapy, n (%)			0.8248
None	5 (4)	3 (7)	
Thrombolysis	9 (8)	2 (5)	
POBA	20 (18)	7 (17)	
Stent	80 (70)	30 (71)	
TIMI 3, n (%)	112 (98)	40 (95)	0.2935
Peak creatine kinase (IU/L)[a]	2257 (1179–4300)	1315 (759–2800)	0.0643
Peak creatine kinase-MB (IU/L)[a]	170 (84–305)	166 (63–364)	0.4253

HDL, high-density lipoprotein; LAD, left artery descending; LCx, left circumflex artery; POBA, plain old balloon angioplasty; RCA, right coronary artery; TIMI, thrombolysis in myocardial infarction; VD, vessel disease.
[a] Median (25–75th percentiles).

were transformed logarithmically before multivariate analysis to fulfill the conditions required for this type of analysis. Cox proportional-hazards analysis was performed to assess the short-term prognosis (1-year MACE post-AMI). We also assessed the independent predictors of MACE during the entire follow-up period using multivariate logistic regression analysis including variables that were significantly associated with MACE in univariate analysis in men and in women, respectively. These analyses were performed using SPSS (SPSS Inc., Chicago, Illinois). Statistical significance was defined as $p<0.05$.

The best cutoff for predicting MACE after AMI was defined as that which yielded the highest product of sensitivity and specificity [17]. To determine the best cutoff level for plasma adiponectin, a receiver operating characteristic (ROC) curve was generated by using the computer program LABROC5 provided by Metz et al. [18].

3. Results

3.1. Clinical background, outcomes and classical predictors of MACE

Table 1 shows the clinical characteristics of men and women with AMI. Women were significantly older and less likely to be smokers, and had significantly higher HDL cholesterol levels and lower body mass index (BMI) than men. Furthermore, the time between the onset of AMI and hospitalization was significantly longer in women. Patients were followed for a mean period of 663 ± 384 days (range: 28–1623 days). During the entire follow-up period, MACE was observed in 13 men (cardiac-related death [$n=1$], recurrent myocardial infarction [$n=2$], unstable angina [$n=7$], heart failure [$n=3$]) and in 10 women (cardiac-related death [$n=2$], recurrent myocardial infarction [$n=1$], unstable angina [$n=5$], heart failure [$n=2$]). Fig. 1 shows event-free survival curves of patients after AMI. Compared with men for post-AMI MACE, the hazard ratio for women was 2.4 after non-adjustment (95% CI: 1.018–5.608, $p=0.0454$) and 5.6 after adjustment for prevalent variables such as age, time to hospitalization, hypertension, diabetes mellitus, total cholesterol, triglyceride, HDL cholesterol, smoking, body mass index, Killip class, vessel involvement, and peak CK (95% CI: 1.029–30.984, $p=0.0463$). In all women, MACE

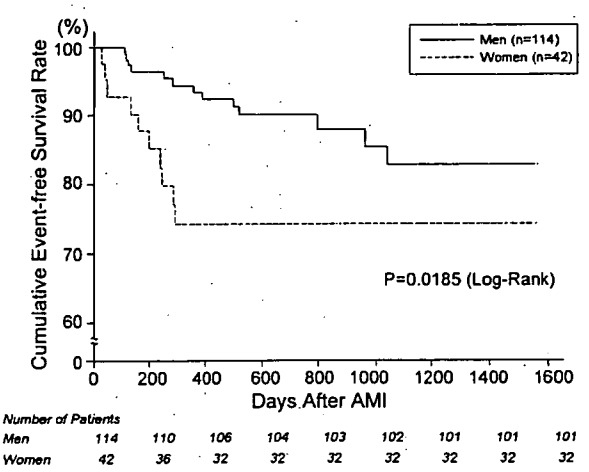

Fig. 1. Event-free survival after acute myocardial infarction (AMI) according to sex.