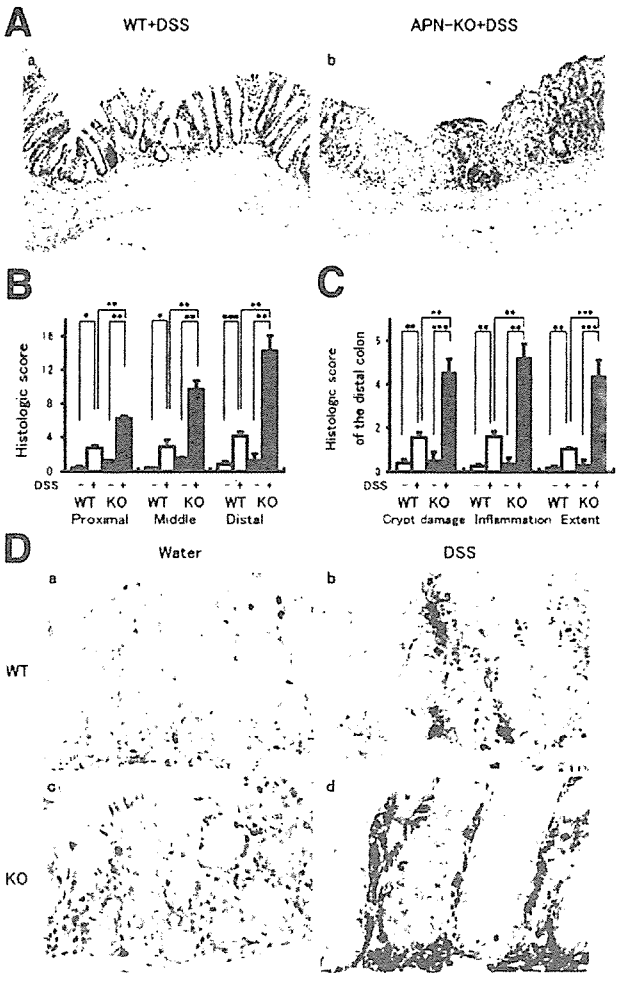

Figure 2. Histopathologic analysis. Representative photomicrographs of paraffin-embedded, H&E-stained sections of the distal colon in DSS-treated mice. (*A* [a]) Colonic sections of DSS-treated WT mice (original magnification 100×). (*A* [b]) Colonic sections of DSS-treated APN-KO mice (original magnification 100×). Histopathologic score. Histopathologic changes were scored in a blinded fashion, as described in Materials and Methods. (*B*) Total histologic score in the proximal colon, middle colon, and distal colon. (*C*) Histopathologic score of crypt damage, severity, and extent of inflammation in the distal colon. Data are mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. WT + water, n = 5; APN-KO + water, n = 5; WT + DSS, n = 6; APN-KO + DSS, n = 6. (*D*) Immunohistochemical staining for macrophages in the colon using F4/80 antibody. [a] WT + water. [b] WT + DSS. [c] APN-KO + water. Macrophages are localized predominantly in the lamina propria of the colonic mucosa of control mice. [d] APN-KO + DSS. The number of F4/80-positive macrophages in the colon of DSS-treated APN-KO mice is larger than that of DSS-treated WT mice (original magnification 100×).

adhesion molecules (E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1), and chemokines (MIP-2 and monocyte chemoattractant protein [MCP]-1) were all significantly higher in the colonic tissues of DSS-treated APN-KO mice compared with DSS-treated WT mice (Figure 3).

Adenovirus-Mediated Adiponectin Supplementation Attenuates DSS-Induced Colitis

To investigate the effect of adiponectin supplementation on the severity of DSS-induced colitis, we constructed recombinant adenovirus-producing mouse adiponectin. WT and APN-KO mice were injected with Ad-β-gal or Ad-APN at 2 days before DSS treatment. Seventeen days after the virus injection, adiponectin protein was detected by immunoblotting analysis in the liver and plasma of Ad-APN-injected APN-KO mice, whereas no expression was detected in Ad-β-gal-injected APN-KO mice (Figure 4*A*). Ad-APN injection significantly ameliorated DSS-induced weight loss and strongly suppressed the DAI on day 11 in both WT and APN-KO mice compared with control Ad-β-gal injection (Figure 4*B* and *C*). Furthermore, Ad-APN injection also prevented shortening and thickening of the colonic mucosa (Figure 4*D* and *E*).

We also examined the histopathologic response to Ad-APN in WT and APN-KO mice with DSS-induced colitis (Figure 5). Examination of H&E-stained sections of the colon showed that treatment with Ad-APN resulted in the suppression of DSS-induced colitis (Figure 5*A*) and significantly reduced the total histologic score in both WT and APN-KO mice relative to

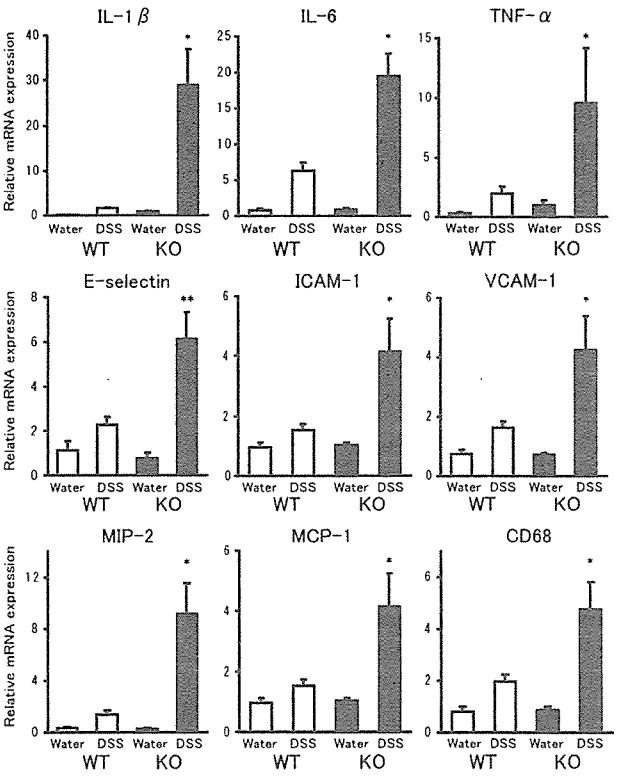

Figure 3. Increased expression of inflammatory-related genes and CD68 during DSS-induced colitis in APN-KO mice. The mRNA expression levels were examined in whole colon tissues of mice on day 15. The levels of the indicated mRNAs were measured by real-time PCR and normalized to the level of 18S ribosomal RNA. Data are expressed as mean ± SEM. $^{*}P < .05$ vs WT + DSS. $^{**}P < .01$ vs WT + DSS. WT + water, n = 5; APN-KO + water, n = 5; WT + DSS, n = 6; APN-KO + DSS, n = 6.

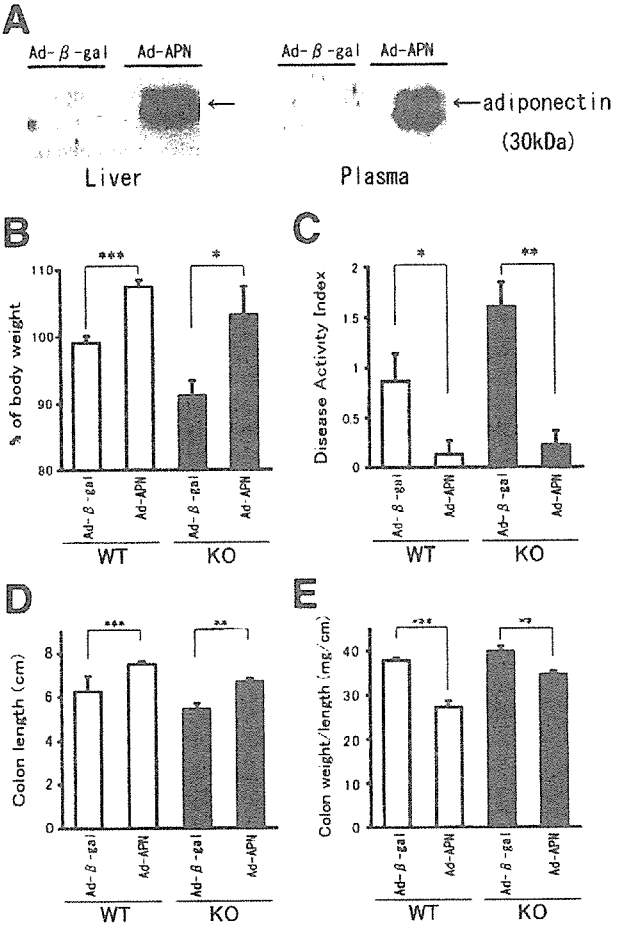

Figure 4. Adenovirus-mediated supplementation of adiponectin in both WT and APN-KO mice attenuated the severity of DSS-induced colitis. Adenoviral vectors expressing adiponectin (Ad-APN, total, n = 5) or β-galactosidase (Ad-β-gal, n = 5) were delivered intravenously through the jugular vein 2 days before DSS treatment. (*A*) Immunoblotting analysis for adiponectin in the liver and plasma of APN-KO mice injected with Ad-APN. (*B*) Changes in body weight (%) determined on day 15 after DSS treatment. (*C*) DAI was determined on day 11 after DSS treatment. (*D*) Colon length. (*E*) Colon weight-to-length ratio. Data are expressed as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

Ad-β-gal–injected mice (Figure 5*B*). These results demonstrated that adiponectin supplementation could protect the development of DSS-induced colitis.

Expression of Adiponectin Receptors in the Mouse Colonic Tissue and Human Colonic Epithelial Cell Lines

The mRNA expression levels of adiponectin receptors adipoR1 and adipoR2 were measured in the gut of mice by RT-PCR. As shown in Figure 6*A*, both receptors were expressed throughout the gut; in particular, adipoR1 was abundantly expressed in the colon and cecum at levels similar to those in the skeletal muscle. To determine the effect of adiponectin on intestinal epithelium, we confirmed the expression of these receptors in human intestinal epithelial cell lines (Caco-2, T-84, and SW-480 cells) by RT-PCR (Figure 6*B*).

Adiponectin Inhibits LPS-Induced IL-8 Production in HT-29 Cells

Changes in LPS-induced IL-8 mRNA expression and secretion by adiponectin are shown in Figure 6*C* and *D*, respectively. LPS stimulation increased IL-8 production in HT-29 cells by 3-fold at the mRNA and protein levels. This effect was abolished when these cells were pretreated with adiponectin, whereas adiponectin alone exhibited no effect on IL-8 production.

Evaluation of TNBS-Induced Colitis

To evaluate the effect of adiponectin deficiency on intestinal inflammation in a distinct model, colitis was induced with TNBS. Body weight changes and the survival rate were used to assess clinical findings. TNBS-treated WT and APN-KO mice showed a similar pattern of body weight changes during the experimental course (Figure 7*A*), and we found no signifi-

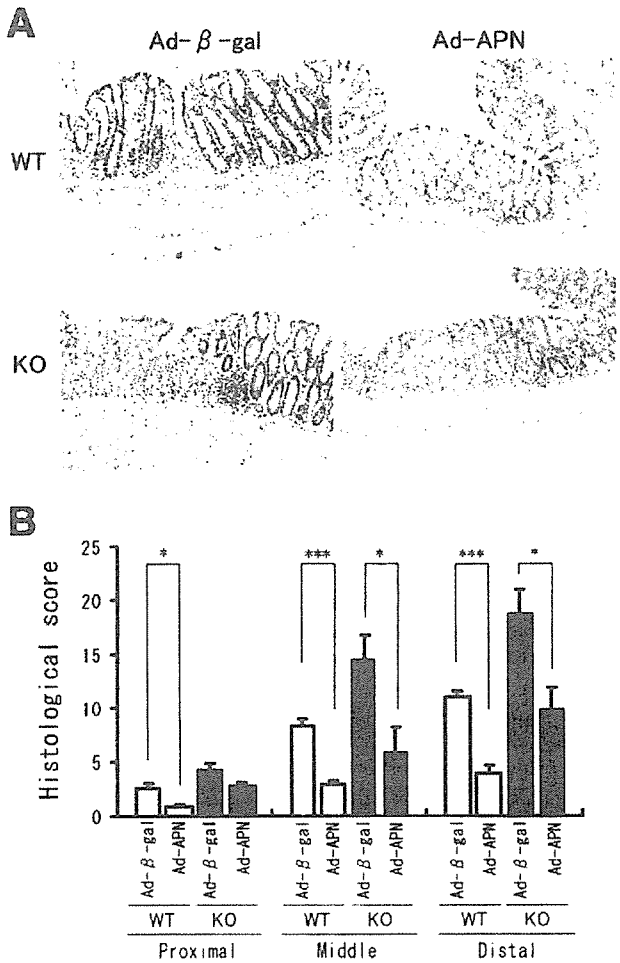

Figure 5. Histopathology and histopathologic score of DSS-induced colitis in mice treated with adenovirus-mediated supplementation of adiponectin. (*A*) Representative H&E-stained sections of the distal colon from Ad-β-gal– or Ad-APN–treated WT and APN-KO mice (original magnification 100×). (*B*) Total histopathologic score in the proximal, middle, and distal sections of the colon. Data are expressed as mean ± SEM. $^{*}P < .05$, $^{***}P < .001$.

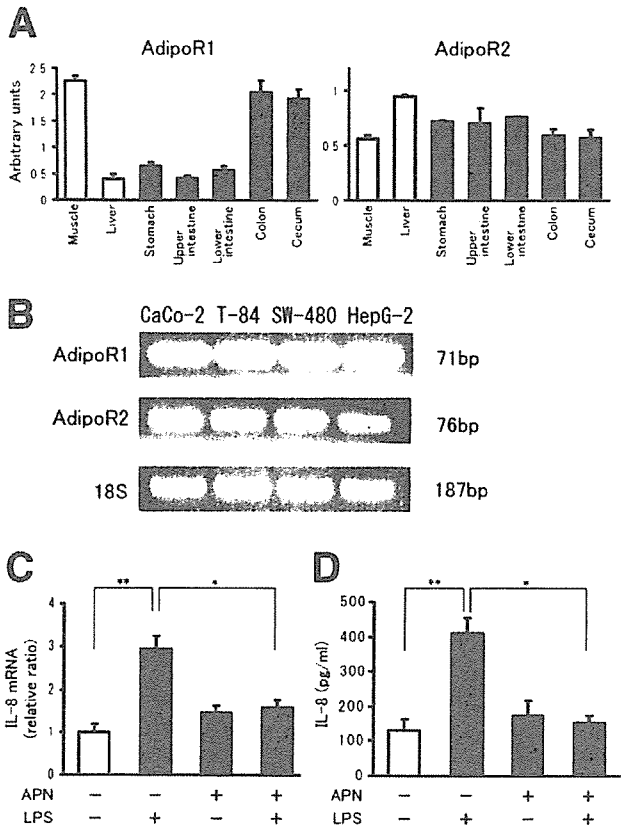

Figure 6. Expression of adiponectin receptors and the effect of adiponectin on human intestinal epithelial cell lines. (*A*) Expression of adiponectin receptors adipoR1 and adipoR2 through the gut of mice was examined by RT-PCR and normalized to the level of 18S ribosomal RNA. The skeletal muscle and liver were used as controls. Data are representative of 3 experiments (means ± SEM). (*B*) Expression of transcripts for adiponectin receptors adipoR1 (71–base pair fragments) and adipoR2 (76–base pair fragments) in the human intestinal epithelial cell lines (CaCo-2, T-84, SW-480) and control HepG-2 cells. (*C*) Adiponectin inhibited LPS-induced IL-8 mRNA expression in HT-29 cells. IL-8 mRNA was determined by RT-PCR and normalized to the level of cyclophilin RNA. Data are representative of 4 experiments (means ± SEM). $^{*}P < .05$, $^{**}P < .01$. (*D*) Adiponectin inhibited LPS-induced IL-8 release from HT-29 cells. The concentration of IL-8 in the media was measured using enzyme-linked immunosorbent assay. Data are representative of 3 experiments (means ± SEM). $^{*}P < .05$, $^{**}P < .01$.

cant differences in the survival rate (Figure 7*B*). Moreover, we assessed histologic findings of the distal colonic tissue. The colon sections of both control WT and APN-KO mice did not show any signs of inflammation (Figure 7*C* [a and d]).

The histologic analysis confirmed that both WT (Figure 7*C* [b and c]) and APN-KO mice (Figure 7*C* [e and f]) were susceptible to TNBS, showing inflammatory infiltrates, loss of cryptal cells, and thickening of the colonic wall.

Discussion

In the present study, we showed that adiponectin exerts protective effects in DSS-induced murine colitis. APN-KO mice developed much more severe colitis by treatment with a very low concentration of DSS compared with WT mice, accompanied by an increase in the colonic mRNA expression of chemokines, such as MCP-1 and MIP-2, and infiltrated leukocytes, including macrophages; in addition, adenovirus-mediated supplementation of adiponectin significantly attenuated disease severity. We showed that adiponectin inhibits LPS-induced IL-8 production in HT-29 cells, suggesting that adiponectin has a direct anti-inflammatory effect on intestinal epithelial cells.

Intestinal epithelial cells regulate the absorption of nutrients and serve as a critical barrier to the external environment. These cells also participate actively in the mucosal immune and inflammatory responses by producing cytokines and chemokines responding to various stimuli, including certain pathogens.[22,23,31] IL-8 in particular plays a central role in the initiation and maintenance of inflammatory responses in the pathogenesis of human inflammatory bowel disease.[32] Moreover, the production of MIP-2, which acts in the mouse in a manner similar to IL-8 in humans, from the intestinal epithelium has been reported as an important mechanism of DSS-induced

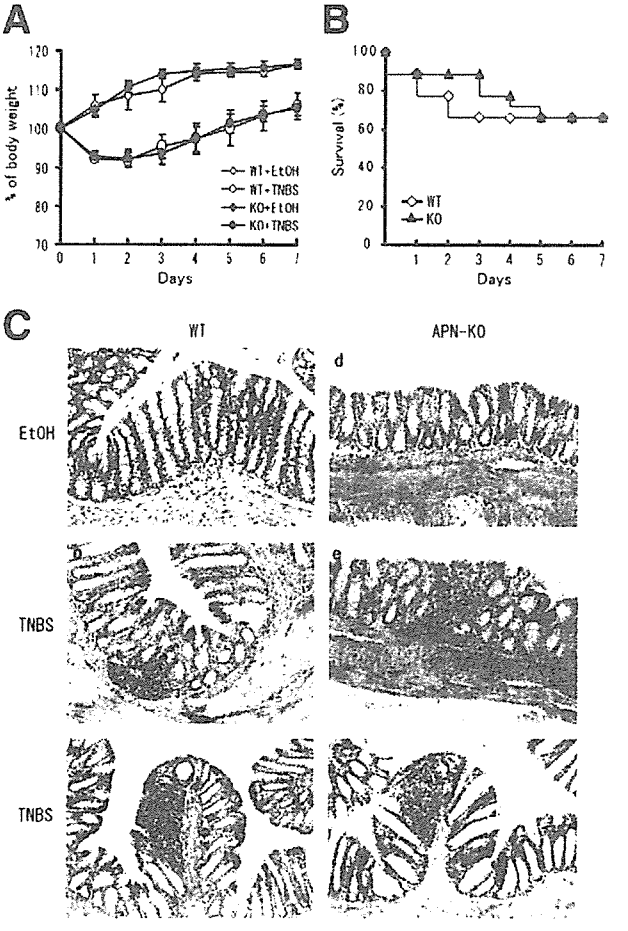

Figure 7. Evaluation of TNBS-induced colitis. (*A*) Changes in percentage of body weight. Control WT, n = 5; control APN-KO, n = 5; WT + TNBS, n = 6; APN-KO + TNBS, n = 6. (*B*) Survival rate, n = 9 per experimental group. (*C*) Representative photomicrographs of paraffin-embedded, H&E-stained sections of the distal colon in TNBS-induced colitis (original magnification 100×). [a] Control WT mice. [b and c] TNBS-treated WT mice. [d] Control APN-KO mice. [e and f] TNBS-treated APN-KO mice.

mucosal inflammation.[24] In the present study, we showed that the mRNA expression of chemokines such as MIP-2 and MCP-1 significantly increased in the colonic tissue of DSS-treated APN-KO mice and that adiponectin inhibited LPS-induced IL-8 production in HT-29 cells. These results suggest that adiponectin directly targets intestinal epithelial cells and attenuates pathogen-induced production of chemokines in the intestinal epithelium in mice with colitis. In APN-KO mice, increased release of chemokines from epithelial cells should result in recruitment of neutrophils and macrophages; moreover, increased release of proinflammatory cytokines, such as IL-1β, IL-6, and tumor necrosis factor α, from these infiltrated cells should worsen the mucosal inflammatory changes. Recently, Yamaguchi et al[33] showed that an antibody against adipoR1 blocked the suppressive effect of adiponectin on LPS-induced activation of nuclear factor κB in RAW264 cells, suggesting that adipoR1 plays an important role in adiponectin signaling for exerting the anti-inflammatory effect and the metabolic effect. We showed that adipoR1 was most abundantly expressed in the colon throughout the gastrointestinal tract of mice. These results suggest that adipoR1 could also play a role in mediating the suppressive effect of adiponectin on the pathogen-induced activation of nuclear factor κB, resulting in reducing production of chemokine IL-8 (MIP-2), which is recognized as a target gene of nuclear factor κB,[34,35] in intestinal epithelial cells. However, the precise intracellular signaling pathway mediating the effect of adiponectin remains to be elucidated.

Recruited immune cells, including neutrophils, macrophages, or lymphocytes, can also be involved in the inflammatory responses through the reciprocal interaction with intestinal epithelium.[22,23] According to the previous reports, crucial immune cells in the inflammatory response are different between DSS- and TNBS-induced colitis models; macrophages substantially contribute to this response in the DSS-induced colitis model,[30,36] whereas T cells play a pivotal role in the TNBS-induced colitis model.[37,38] Our current studies showed that APN-KO mice exhibited enhanced susceptibility to DSS-induced colitis, but not to TNBS-induced colitis, suggesting adiponectin might preferentially inhibit macrophages rather than T cells among immune cells that participate in the pathogenesis of colitis. In this regard, we and others previously showed that adiponectin exerted the anti-inflammatory effects on macrophages by suppressing phagocytic activity and the production of proinflammatory cytokines, accompanied by induction of anti-inflammatory cytokines, IL-10, and IL-1 receptor antagonists.[18-20] These current and previous data suggest that the inhibitory effects on macrophages may also be essential for protective roles of adiponectin from colitis. However, in our experiments of TNBS-induced colitis, single administration of TNBS introduced acute epithelial damage and subsequent inflammation; these changes did not persist. To establish whether adiponectin affects T-cell-mediated colitis, further analysis will be required using a chronic colitis model such as IL-10 KO mice.

In the present study, we showed that treatment with 2.5% DSS resulted in delayed recovery from severe colitis and a worsened survival rate in APN-KO mice compared with WT mice. In this regard, we demonstrated previously that angiogenic repair of ischemic hind limbs was impaired in APN-KO mice compared with wild-type mice and that adenovirus-mediated supplementation of adiponectin accelerated angiogenic repair in both APN-KO and WT mice, indicating that adiponectin promotes angiogenesis in response to tissue ischemia.[39] These data suggest that adiponectin possesses an enhancing effect on the remodeling of damaged colonic tissue, which might be due to promotion of angiogenesis as well as attenuation of prolonged inflammation in the colonic tissue.

In patients with Crohn's disease, the increased accumulation of fat tissue surrounding the intestine has been observed.[40] Recently, we reported that the protein levels of adiponectin in this fat tissue negatively correlated to disease severity in patients with Crohn's disease,[21] which suggests that adiponectin released by visceral or mesenteric fat tissue is likely to have a role in the pathogenesis of colitis. In the current study, we showed that adiponectin should exhibit suppressive effects on DSS-induced colitis, probably through inhibiting the proinflammatory actions of epithelial cells and macrophages in the colon. These current findings further support the hypothesis of a significant role of a visceral fat-derived adiponectin on the epithelial immune system of intestine. It is conceivable that a decrease of adiponectin in visceral fat tissue, which is seen in obesity or other physiologic situations, might be causally related to the development of colitis.

To summarize, adiponectin exerts protective effects against DSS-induced colitis, probably due to the suppression of chemokine production in intestinal epithelial cells and the following inflammatory responses, including infiltration of macrophages and release of proinflammatory cytokines.

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Visfatin in adipocytes is upregulated by hypoxia through HIF1α-dependent mechanism

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Abstract

Obesity is associated with metabolic disorders, such as insulin resistance. Visfatin is an adipose-derived secretory factor to exert insulin-mimetic effects. Plasma visfatin levels and mRNA levels of visfatin in adipose tissues are increased in obesity. However, the mechanism that mediates induction of visfatin mRNA in adipose tissue of obesity remains unknown. Recent studies have reported that fat tissue is hypoxia in obesity. In this study, we investigated the effects of hypoxia on mRNA expression of visfatin in adipocytes. Hypoxia increased visfatin mRNA expression. Desferoxamine and Cobaltous chloride, two hypoxia mimetic compounds, also increased visfatin mRNA levels. Dimethyloxallyl glycine, a stabilizer of hypoxia-inducible factor 1α (HIF1α), mimicked the hypoxia-mediated upregulation of visfatin, and YC1, an inhibitor of HIF1 cancelled the hypoxia-induced upregulation of visfatin mRNA. We identified two functional hypoxia responsive elements (HRE) in mouse visfatin promoter. Hypoxic treatment and overexpression of HIF1α increased the promoter activity, and mutation of the HRE blunted hypoxia-induced activation of visfatin promoter. Our results suggest that visfatin mRNA expression is upregulated in the fat tissue of obesity through the activation of HIF1α pathway due to hypoxia.

Keywords: Visfatin; Hypoxia; HIF1α; Obesity; Metabolic syndrome

Recent studies have revealed that adipose tissue is not only an energy-storaging organ but it produces and secretes a variety of bioactive molecules called adipocytokines, including tumor necrosis factor (TNF), leptin, resistin, and plasminogen activator inhibitor type-1 (PAI-1) [1–4]. Dysregulated production of adipocytokines is associated with the pathophysiology of obesity-related metabolic diseases [5–9].

We reported visfatin as a novel adipocytokine implicated in abdominal visceral fat obesity [10]. Visfatin is identical to pre-B cell colony-enhancing factor (PBEF), which is originally cloned as a growth factor, enhancing the effect of IL-7 and stem cell factor on early stage B cells [11]. In obese mice, visfatin mRNA expression was increased in both mesenteric and subcutaneous fat depots, compared to those in control mice, and plasma visfatin levels correlated with the amount of visceral fat in human subjects [10]. Visfatin showed insulin-mimetic effect to lower plasma glucose levels in mice. In vitro, visfatin treatment increased glucose uptake in 3T3-L1 adipocytes and L6 myocytes, and suppressed glucose release from H4IIEC3 hepatocytes. Moreover, recombinant visfatin protein directly binds to and stimulates the insulin receptor [10]. These data suggest that visfatin is an adipocyte-derived factor associated with visceral fat obesity. However, the precise mechanisms responsible for the upregulation of visfatin expression in accumulated fat have not been elucidated.

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Obesity is attributed to hypertrophy and hyperplasia of adipocytes. Adipocytes become hypertrophic during development of obesity and their size increases up to 140–180 μm in diameter [12]. The size of hypertrophic adipocytes is limited because the diffusion limit of oxygen into cell is at most 100 μm [13]. Therefore, hypertrophic adipocytes are provided with low oxygen tension. Several reports showed hypoxia in fat tissues of obesity [14–16]. In the presence of hypoxia, cells must respond by coordinated expression of numerous genes to ensure adaptation. Hypoxia-inducible genes include erythropoietin (EPO) [17], vascular endothelial growth factor (VEGF) [18], glucose transporter 1 (GLUT1) [19], lactate dehydrogenase A [20], and phosphoglycerate kinase 1 [21]. An important and well-characterized key regulator of the adaptive response to low oxygen tension is hypoxia-inducible factor-1 (HIF1) [22], a transcription factor that accumulates during hypoxia. HIF1 is a heterodimer composed of HIF1α and HIF1β subunits, and its activity is tightly regulated by cellular oxygen tension [23], and the hypoxia response regions of above genes have one or more binding sites for HIF1. Previous report demonstrated that HIF1 is elevated in adipose tissue of human obese subjects and is decreased following weight loss [16].

In the present study, we found that exposure of 3T3-L1 adipocytes to hypoxia-induced upregulation of visfatin mRNA transcription, and hypoxic induction of visfatin mRNA expression was mediated by HIF1 and two hypoxia responsive elements (HRE) existing in the promoter region of visfatin gene.

Materials and methods

Materials. Desferoxamine (DFO) and Cobaltous chloride ($CoCl_2$) were purchased from Sigma (St. Louis, MO, USA). Dimethyloxallyl glycine (DMOG) was purchased from Cayman (Ann Arbor, MI, USA). YC1 was purchased from BIOMOL (Butler Pike, PA, USA). Bacterial artificial chromosome (BAC) clone (RPCI-23 NM BAC clone# 26B22) was purchased from Invitrogen (Carlsbad, CA, USA). pCEP4-human-HIF1α was purchased from ATCC Johns Hopkins special collection (MBA-2, provider investigator: G.L. Semenza).

Plasmids. Mouse visfatin promoters were subcloned using the *Escherichia coli*-based bacterial artificial chromosome (BAC) recombineering. Retrieval cassettes were PCR-amplified from pBSIIKS+ vector, using mVisfatin+58 bp-BglII-pBS (forward) primer and series of mVisfatin-MluI-pBS (reverse) primers as follows. Nucleotides in italics are homologous to the targeted sequence. mVisfatin+58 bp-BglII-pBS (forward), 5′-*TGCGGCAGAAGCCGAGTTCAACATCCTGCTGGCCACCG ACTCGTACAAGG*AGATCTGCTTTTGTTCCCTTTAGTGAGG-3′. mVisfatin-MluI-2028 bp-pBS (reverse), 5′-*TATGAACTAAGCAGTA CCCCGGAGCTCTTGTGTCTAGCTGCATATGTATC*ACGCGTCA ATTCGCCCTATAGTGAGTCGTATTAC-3′. mVisfatin-MluI-1007 bp-pBS (reverse), 5′-*ATTAAATCGTGGGGGAAAGAGCTAGTAACCT CCCAAATAAGCGAAGTGAC*ACGCGTCAATTCGCCCTATAG TGAGTCGTATTAC-3′. mVisfatin-MluI-510 bp-pBS (reverse), 5′-*G GAGTCTCTTTGCCCGCCCCAACCCGGACCTTC CTCTTGTCCT ACCCAGT*ACGCGTCAATTCGCCCTATAGTGAGTCGTATTA C-3′. mVisfatin-MluI-330 bp-pBS (reverse), 5′-*GCGCAGCGCAGGGGC GGGGGGGACTGAGGAGGACGTGAGGCACGCGCTCT*ACGCGT CAATTCGCCCTATAGTGAGTCGTATTA C-3′. mVisfatin-MluI-308 bp-pBS (reverse), 5′-*GGGGCGGGGCACGGCTGCGCGTGCGC AGCGCAGGGGCGGGGGGGACTGAG*ACGCGTCAATTCGCCCT ATAGTGAGTCGTATTAC-3′. mVisfatin-MluI-222-+58 bp-BglII-pGL3basic plasmid was amplified by PCR.

Construction of mutated plasmids. −308 bp to +58 bp of mouse Visfatin promoter was excised by *Dde*I and *Bgl*II site of m-330 bp Luc promoter. This fragment and a series of mutant Oligos were inserted into *Mlu*I and *Bgl*II sites of pGL3-basic plamids. Sequences of oligos are follows: Mutant1 (forward) 5′-CGCGTAGAGCAAAGCCTCACGTCCT CC-3′ , Mutant1 (reverse) 5′-TGAGGAGGACGTGAGGCTTTCGC TCTA-3′, Mutant2 (forward) 5′-CGCGTAGAGCGCGTGCCTCTTT TCCTC-3′, Mutant2 (reverse) 5′-TGAGGAGGAAAAGAGGCACGC GCTCTA-3′, Mutant3 (forward) 5′-CGCGTAGAGCGAAAGCC TCTTTTCCTCC-3′, Mutant3 (reverse), 5′-TGAGGAGGAAAAGAGG CTTTCGCTCTA-3′. Mutant4 (forward) 5′-CGCGTAAAACGCGT GCCTCACGTCCTCC-3′, Mutant4 (reverse) 5′-TGAGGAGGACGT GAGGCACGCGTTTTA-3′, Mutant5 (forward) 5′-CGCGTAGAGC GCGTGAAACACGTCCTCC-3′, Mutant5 (reverse) 5′-TGAGGAGGA CGTGTTTCACGCGCTCTA-3′, Mutant6 (forward) 5′-CGCGTAGA GCGCGTGCCTCACGTCAAAC-3′, Mutant6 (reverse) 5′-TGAGTTT GACGTGAGGCACGCGCTCTA-3′.

RNA preparations from 3T3-L1 adipocytes. 3T3-L1 cells were maintained in DMEM containing 10% FBS. For differentiation, cells (3–7 days after reaching confluence) were cultured for 2 days with 10% FBS-supplemented DMEM containing 5 μg/ml of Insulin, 0.5 mmol/L 1-methyl-3-isobutyl-xanthine, and 1 μmol/L dexamethasone. The cells were further incubated with DMEM containing 10% FBS to differentiate into adipocytes. Total RNA was extracted from differentiated 3T3-L1 adipocytes (on day 7 after incubation of differentiation) using RNA-STAT-60 (Tel-Test, Friendswood, TX, USA) according to the manufacture's protocol.

Quantitative real-time PCR. The cDNA was synthesized using the ThermoScript RT-PCR system (Invitrogen). Real-time PCR was performed on LightCycler system used the FastStart DNA Master SYBR Green I (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Sequences of primers used for real-time PCR were as follows: visfatin, 5′-GGCCACAAATTCCAGAGAACAG-3′ and 5′-CCAAAT GAGCAGATGCCCCTAT-3′; VEGF, 5′-TCTCT TGGGTGCACTGG ACC-3′ and 5′-GTTACAGCAGCCTGCACAGC-3′; 36B4, 5′-GCTC CAAGCAGATGCAGCA-3′ and 5′-CCGGATGTGAGGCAGCAG-3′. The levels of visfatin and VEGF mRNA were normalized relative to the amount of 36B4 mRNA.

Transfection studies of 3T3-L1 cells. On day 7 after incubation of differentiation, the media of 3T3-L1 cells in 6-well plates were changed to OPTI-MEM (Invitrogen), and the cells were transfected with luciferase reporter plasmids using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Transfection was performed 1 μg pCMX-β-gal (internal standard) along with 2 μg pGL3-basic plasmid containing mouse visfatin promoter or pGL3-basic plasmid alone. Four hours later, the media were changed to DMEM containing 10% FBS, then cells were exposed to hypoxia condition in airtight chamber that was flushed with a gas mixture of 5% CO_2 and 95% N_2 or normoxia condition that was humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After 24 h incubation, luciferase reporter assays were performed using Luciferase Assay system (Promega, Madison, WI ,USA). Luciferase values were normalized by an internal β-galactosidase control and expressed as the relative luciferase activity.

Overexpression of HIF1α assays in 3T3-L1 cells. On day 7 after incubation of differentiation, the media of 3T3-L1 cells in 6-well plates were changed to OPTI-MEM (Invitrogen), and the cells were transfected with luciferase reporter plasmids using Lipofectamine 2000 reagent (Invitrogen) according to the manufactures's protocol. Transfection was performed with 2 μg pCEP4- HIF1α and 1 μg pCMX-β-gal (Internal standard) along with 2 μg of pGL3-basic plasmid containing mouse visfatin promoter or pGL3-basic plasmid alone. Four hours later, the media were changed to DMEM containing 10% FBS, then cells were incubated at normoxia condition. Forty hours later, luciferase reporter assays were performed using Luciferase Assay System (Promega).

Statistical analysis. All data are presented as means ± SD and analyzed using unpaired Student's *t* test. For simultaneous multiple comparisons, differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparisons test. *p* values less than 0.05 were considered statistically significant.

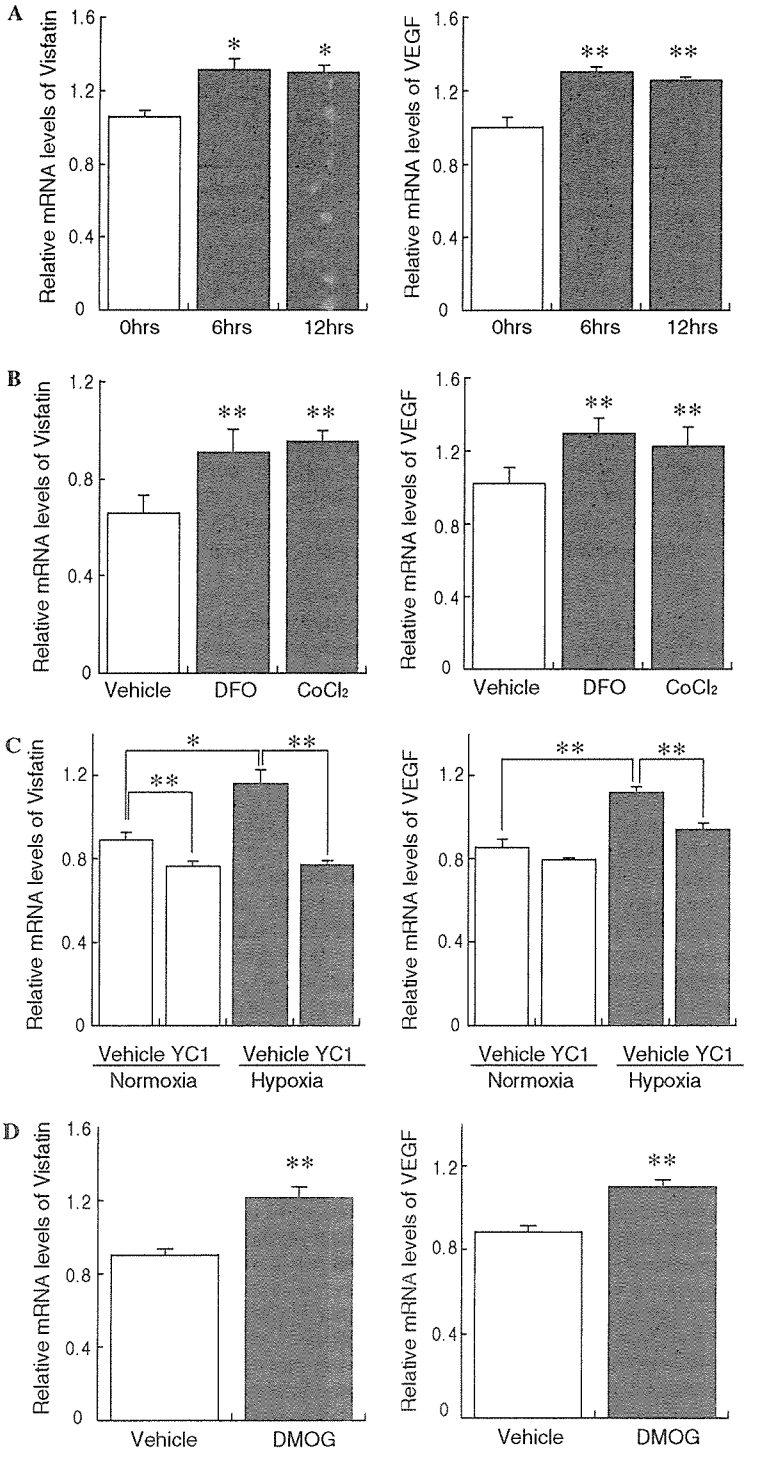

Fig. 1. Effects of hypoxia and HIF1 activity on the expression of visfatin. (A) 3T3-L1 adipocytes were incubated under normoxic (21% O_2) or hypoxic (1% O_2) conditions for the indicated times. mRNA levels of visfatin and VEGF were quantified by real-time PCR and normalized to 36B4. (B) 3T3-L1 adipocytes were exposed to chemical hypoxia with 100 μM Desferoxamine (DFO) or 100 μM Cobaltous chloride ($CoCl_2$) for 24 h. mRNA levels were quantified by real-time PCR. (C) 3T3-L1 adipocytes were treated with 100 μM YC1 under normoxic or hypoxic conditions for 12 h. mRNA levels were quantified by real-time PCR. (D) 3T3-L1 adipocytes were exposed to 0.5 mM DMOG under normoxic condition for 18 h. mRNA levels were quantified by real-time PCR. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control (means ± SD, $n = 3$–5).

Results

Hypoxia increases expression of visfatin in 3T3-L1 adipocytes

To examine the effects of hypoxia on visfatin mRNA expression, 3T3-L1 adipocytes were cultured in 1% O_2. Quantitative real-time PCR demonstrated that visfatin mRNA expression was significantly upregulated in 6 h by about 1.3-fold and in 12 h by about 1.3-fold, in parallel with VEGF mRNA expression, established as a hypoxia responsive gene (Fig. 1A). Next, we tested the effect of hypoxia mimetic compounds, Desferoxamine (DFO) and Cobaltous chloride ($CoCl_2$). Fig. 1B demonstrated that visfatin mRNA expression was significantly increased by these chemicals, in accordance with increased expression of VEGF, in 3T3-L1 adipocytes. To confirm the role played by HIF1, a transcriptional factor that mediates a broad range of adaptation to hypoxia, 3T3-L1 adipocytes were treated with YC1, an inhibitor of HIF1, under hypoxic condition. Hypoxic induction of visfatin mRNA was cancelled by YC1 (Fig. 1C). We next investigated the effect of DMOG, a stabilizer of HIF1 protein (Fig. 1D). Expectedly, treatment of cells with DMOG led to upregulation of visfatin mRNA expression as well as VEGF mRNA even under normoxic condition. Taken together, these data suggest that the HIF1 pathway plays an important role in upregulation of visfatin mRNA.

The visfatin promoter is activated by hypoxia

Promoter database analysis revealed nine putative HREs (−731/−727, −597/−593, −545/−541, −428/−424, −410/−406, −382/−378, −325/−321, −317/−313, and −281/−277) and eight conserved elements (−1550/−1527, −1515/−1491, −1420/−1355, −920/−879, −782/−762, −512/−499, −330/−310, and −284/−275) in −2025 bp promoter region of mouse visfatin gene (Fig. 2A). Among these conserved elements, two contained HRE. One (−330/−310) contained an inverted repeat of two HREs, and the other (−284/−275) contained one reverse HRE. To

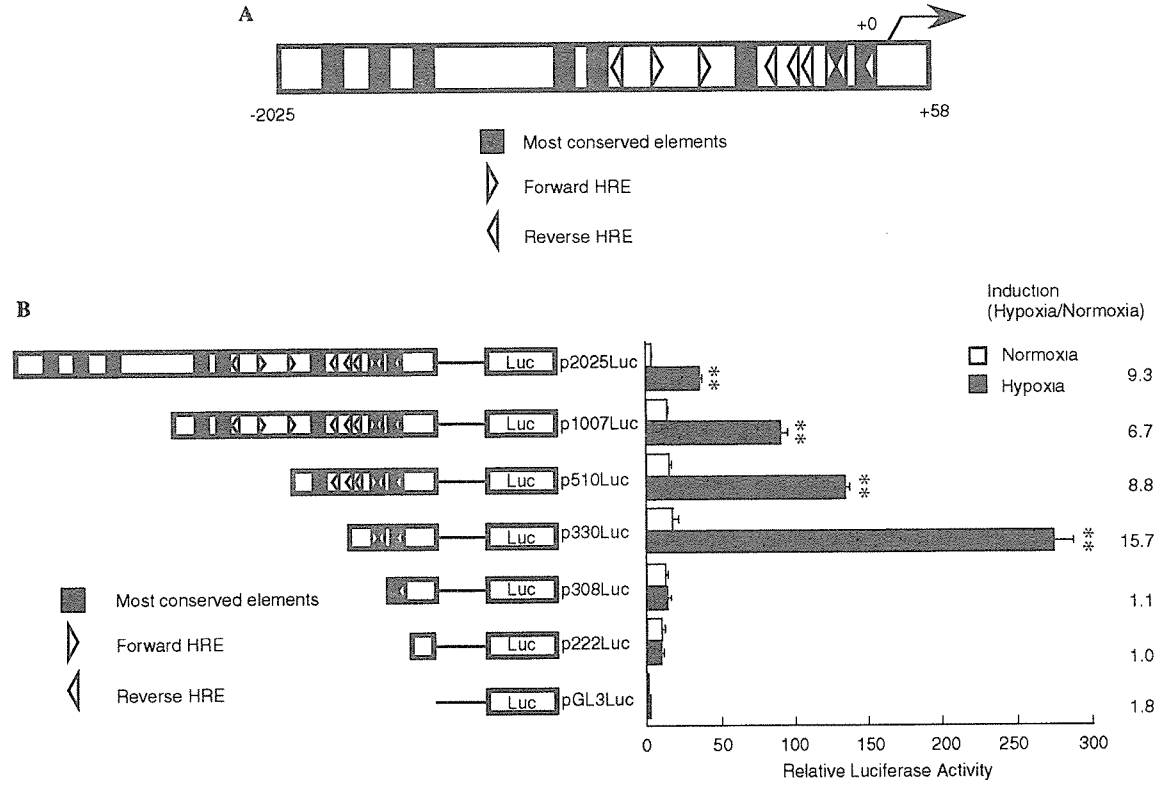

Fig. 2. Deletion analysis of the 5′-flanking region of the visfatin gene and effect of hypoxia upon reporter gene expression. (A) The location of putative HRE sites and most conserved elements within the 2025 bp fragment of the 5′-flanking region of the mouse visfatin gene. Putative HRE sites are defined by the core sequence 5′-A/G-CGTG-3′. Most conserved elements are defined with UCSC Genome browser on mouse Aug. 2005 Assembly, http://genome.ucsc.edu/cgi-bin/hgGateway. The arrow indicates the direction of transcription. Putative HRE sites are indicated by open triangles. Most conserved elements among different species are indicated by black squares. (B) A series of fragments of the 5′-flanking region of the visfatin gene was subcloned into upstream of a luciferase reporter gene as described under Materials and methods. Each of these promoter/reporter constructs was transfected into 3T3-L1 adipocytes and exposed to normoxic (21% O_2) or hypoxic (1% O_2) conditions for 24 h. Luciferase activities were measured and normalized by β-galactosidase activity. Induction indicates the luciferase activity under hypoxia relative to normoxia condition. $^{**}p < 0.01$ versus normoxia (means ± SD, $n = 3$).

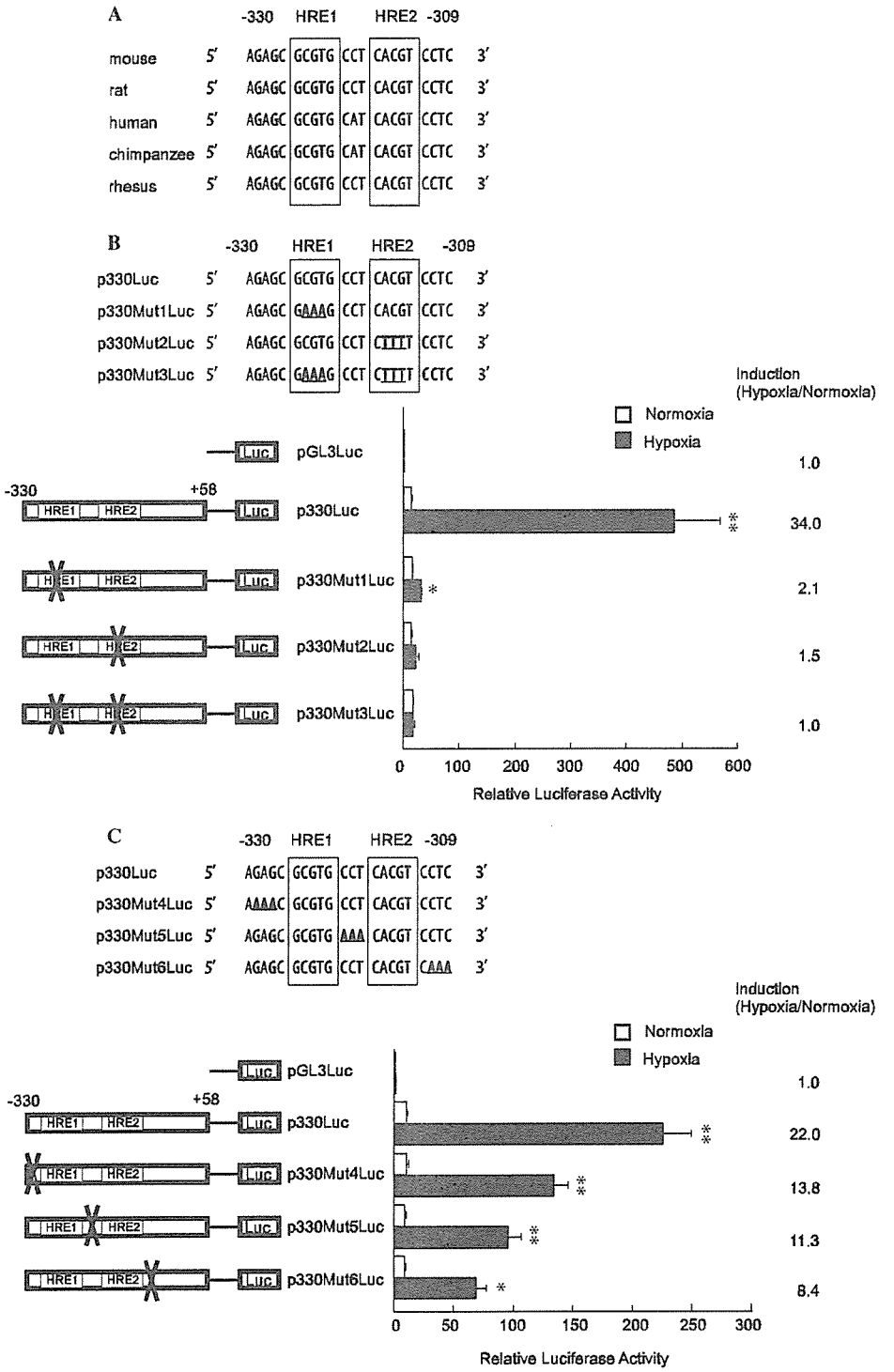

Fig. 3. Effect of HRE site specific mutations on transactivation of the proximal promoter region of the visfatin gene by hypoxia. (A) Conservation of HRE sites in mammalian visfatin genes. (B) 3T3-L1 adipocytes were transfected with p330Luc or mutated promoter constructs (p330Mut1Luc/330 bp promoter with a mutation of HRE1 site, p330Mut2Luc/330 bp promoter with a mutation of HRE2 site, and p330Mut3Luc/330 bp promoter with mutations of HRE1 and HRE2 sites). (C) 3T3-L1 adipocytes were transfected with p330Luc or mutated promoter constructs (p330Mut4Luc/330 bp promoter with a mutation located immediately upstream of HRE1 site, p330Mut5Luc/330 bp promoter with a mutation located between HRE1 and HRE2 sites, and p330Mut6Luc/330 bp promoter with a mutation located immediately downstream of HRE2 site). After transfection, cells were incubated under normoxic (21% O_2) or hypoxic (1% O_2) conditions for 24 h. Luciferase activities were measured and normalized by β-galactosidase activity. Induction indicates the luciferase activity under hypoxia relative to normoxia condition. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus normoxia (means ± SD, $n = 3$).

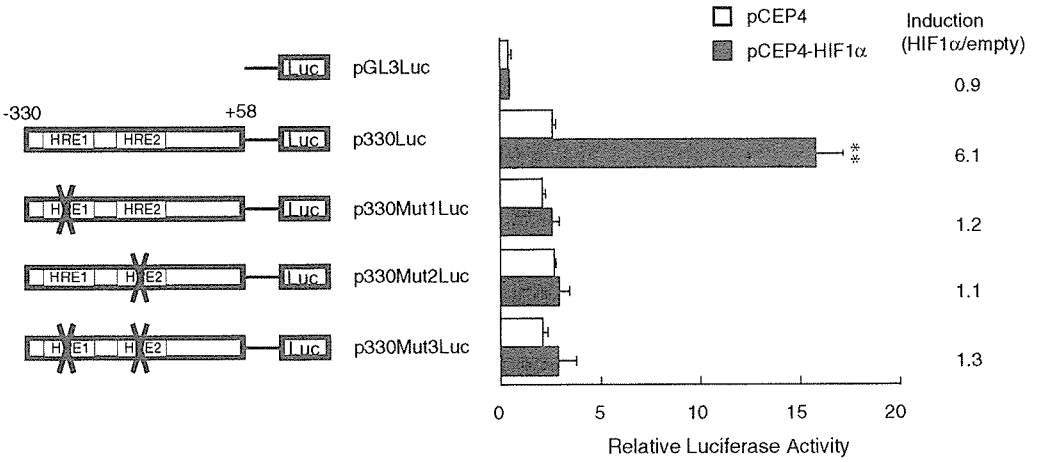

Fig. 4. Effect of HIF1α expression on transcription of the visfatin proximal promoter. 3T3-L1 adipocytes were cotransfected with the reporter construct (p330Luc, p330Mut1Luc, p330Mut2Luc, or p330Mut3Luc) and an expression vector of HIF1α. After transfection, cells were incubated for 48 h under normoxia (21% O_2). Luciferase activities were measured and normalized by β-galactosidase activity. Induction indicates the luciferase activity with the HIF1α expression vector relative to the empty vector. $^{**}p < 0.01$ versus the empty expression vector, pCEP4 (means ± SD, $n = 3$).

determine the HRE responsible for hypoxic induction of visfatin, luciferase activity driven by series of deletion constructs of visfatin promoter was measured in 3T3-L1 adipocytes under normoxic and hypoxic conditions (Fig. 2B). The luciferase activities driven by −2025, −1007, −510, and −330 bp of visfatin promoters were induced by 6.7 – 15.7-fold under hypoxic condition, but deletion of the sequence between −330 and −309 bp abolished the hypoxic responsiveness of visfatin promoter. This region contained an inverted repeat of two HREs with most conserved elements among species (Fig. 2A and B). These data suggest that two HREs located in −330/−310 are required for the hypoxic induction of visfatin promoter.

Both HREs located at −325/−321 and −317/−313 are required for the hypoxia responsiveness of visfatin promoter

A conserved region located at −330/−310 contains two HREs (HRE1: −325/−321, HRE2: −317/−313) and these elements are highly conserved among many species (Fig. 3A). To investigate whether HRE1 and HRE2 are required for the hypoxia responsiveness of visfatin promoter, series of site-directed mutant constructs (Fig. 3B) were transfected into 3T3-L1 adipocytes, and luciferase activities were measured under normoxic and hypoxic conditions. When either HRE1 or HRE2 was mutated (p330Mut1Luc, p330Mut2Luc, respectively), inductions of promoter activity by hypoxia were almost totally abolished (Fig. 3B). Moreover, when both HRE1 and HRE2 were mutated (p330Mut3Luc), the hypoxic responsiveness of p330Luc was completely abolished (Fig. 3B). Next, to investigate whether the loss of hypoxic induction by mutations of HRE1 and HRE2 sites is HRE specific effect, other mutant constructs were transfected into 3T3-L1 adipocytes (p330Mut4Luc with a mutation located immediately upstream of HRE1 site, p330Mut5Luc with a mutation located between HRE1 and HRE2 sites, and p330Mut6Luc with a mutation located immediately downstream of HRE2 site). As shown in Fig. 3C, these mutant constructs significantly responded to hypoxia. Taken together, both HRE1 and HRE2 should play an important role in mediating the hypoxic response of visfatin promoter.

Overexpression of exogenous HIF1α directly increases visfatin promoter activity

To investigate whether HIF1α is capable of transactivating reporter activity directly through HRE1 and HRE2, transient cotransfection experiments were performed under normoxic conditions. As illustrated in Fig. 4, overexpression of HIF1α resulted in significant induction of visfatin promoter activity when 3T3-L1 adipocytes were cotransfected with p330Luc. This HIF1α-mediated induction was totally blunted with p330Mut1Luc, p330Mut2Luc, or p330Mut3Luc in 3T3-L1 adipocytes. These results support the notion that HIF1α directly transactivates visfatin promoter, and this transactivation requires both HRE1 and HRE2.

Discussion

We and others reported that visfatin mRNA expression was increased in adipose tissue of obese subjects [10,24,25], and weight loss reduced plasma visfatin levels in obese subjects [26]. However, mechanism of visfatin mRNA induction in adipose tissues of obesity is not elucidated.

In order to know the regulators for visfatin mRNA expression in obesity, we treated 3T3-L1 adipocytes with various cytokines and conditions to mimic obesity, such as insulin, glucose, TNFα, IL-6, VEGF, Free fatty acids, and H_2O_2, none of which increased visfatin mRNA expression (data not shown). However, we found that visfatin mRNA expression was upregulated in response to hypoxia. Hypoxia was reported to enhance the mRNA expressions

of other adipocytokines, such as leptin and VEGF, through activation of HIF1 pathway in 3T3-F442A adipocytes [27]. Here, we demonstrated that visfatin mRNA expression was upregulated by hypoxia through gene transactivation mediated by HIF1. We have identified two functional HRE sites in 5′-flanking promoter region of visfatin gene, and using luciferase assay, we showed that mutation of these HRE sites totally abolished the induction of promoter activity by hypoxia. This existence of two adjacent HRE sites, arranged as either direct or inverted repeats, has been noted in other hypoxia-regulated genes including human enolase I [20], mouse lactate dehydrogenase A [20], human phosphoglycerate kinase I [21], and human transferrin [28].

VEGF promoter has one HRE site in its 5′-flanking region and functions as a cis-element regulating the hypoxic induction of VEGF. Kimura et al. reported that adjacent sequence located immediately downstream from the functional HRE, named HAS (HIF1 ancillary sequence), is indispensable for VEGF gene induction by hypoxia [29]. The core sequences of the HRE and the HAS form an imperfect inverted repeat with a spacing of eight nucleotides, and changes in spacing between HRE and HAS diminished the promoter activity. In addition, they showed that HAS is widely seen among hypoxia-inducible genes including VEGF and EPO, and some glycolytic enzyme genes [29]. In the current study, the core sequence of hypoxia responsive region of visfatin gene consists of two perfect HRE with a spacing of three nucleotides. We constructed a mutant visfatin promoter with a spacing of eight nucleotides between these two HREs, but the mutant promoter activity was induced by hypoxia similarly to wild type promoter (data not shown). These data suggested that hypoxia responsible region of visfatin gene is unique, distinguished from typical HAS.

Previous studies have demonstrated reduction of blood flow and vasculature network in adipose tissue of obesity [30–34], and recently we demonstrated that WAT of obese mice is hypoxia as confirmed using pimonidazole hydrochloride adduction and lactate concentration (unpublished data). In addition, in morbid obesity, mRNA expression of HIF1α is higher than in lean subjects [16]. These data indicate that adipose tissue of obesity is hypoxic due to decreased blood flow, which leads to activation of HIF1 signaling [35] and induction of visfatin mRNA expression.

It remains to be cleared about physiological significance of increased visfatin expression by hypoxia. Visfatin is reported to be upregulated in tissues of colon cancer [36], and injured lung [37], both of which are hypoxic state, indicating that visfatin may play some roles in the pathogenesis of hypoxia. There are two possible roles of visfatin in hypoxic tissues. The first is to activate cellular glucose-uptake and maintain ATP levels. It is essential for cell survival to maintain ATP production under limited O_2 availability. Hypoxia induces glycolytic enzymes [19] and GLUT1 [38]. Clinical cancers show more glucose uptake in vivo than do normal tissues [39,40], and the disruption of the HIF1 pathway inhibited glucose uptake into tumor tissues in vivo [41]. GLUT1 is a hypoxia-inducible gene with HRE and plays important roles in glucose uptake under hypoxic conditions [38]. Visfatin is another hypoxia-inducible gene with HRE, and through its insulin-mimetic effect, should induce glucose-uptake [10] to maintain cellular ATP levels and adapt cells to hypoxia. The second possible role of visfatin in hypoxic tissues is to induce angiogenesis. Insulin induced angiogenesis is mediated through Akt activation [35]. In hypoxic tissues, elevated visfatin may activate Akt [10] and induce angiogenesis to accommodate disturbed O_2 tension.

In summary, we demonstrated that visfatin mRNA expression is upregulated by hypoxia, and two HRE sites in promoter region of visfatin gene are essential for this effect. Our results suggest that increased visfatin may play some roles in the pathophysiology of hypoxic fat in obesity.

Acknowledgments

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Adiponectin and Renal Function, and Implication as a Risk of Cardiovascular Disease

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The relation among adiponectin, renal function, and incident cardiovascular disease (CVD) in patients with different degrees of renal dysfunction was investigated. In total, 150 subjects were included in this study and followed prospectively for a mean of 32 months. At baseline, median adiponectin levels for chronic kidney disease (CKD) stages 1, 2, 3, 4 and 5, as estimated by creatinine clearance (≥90, 60 to 90, 30 to 60, <30 ml/min), were 3.06, 4.04, 6.43, and 11.9 μg/ml, respectively (p for trend <0.01), and a significant association between adiponectin and CKD stages was also confirmed in multivariate regression analysis (F = 6.2, p <0.001). During follow-up, 31 subjects developed CVD, including myocardial infarction, angina pectoris, stroke, and transient ischemic attack. Gender-specific median values of adiponectin were used to separate the higher group from the lower group, and the Kaplan-Meier curve showed a significantly lower event-free survival rate in the lower adiponectin group (<4.39 μg/ml in men, <6.84 μg/ml in women, chi-square 4.88, p <0.03). The risk factor-adjusted Cox regression showed that an increase in adiponectin per 1 μg/ml was associated with a decrease in the risk of CVD to 0.86 (95% confidence interval 0.75 to 0.96, p = 0.004). In the subgroup with previous ischemic heart disease (IHD; n = 65), a significantly lower event-free survival rate of IHD was also observed in the lower adiponectin group (<4.45 μg/ml in men, <4.49 μg/ml in women, chi-square 3.96, p <0.05). The relative distribution of adiponectin isoforms was examined in patients with severe CKD, and the percentage of the high-molecular-weight form in patients with IHD during follow-up (n = 3) was significantly smaller than that in those without IHD (n = 4, p <0.02). In conclusion, renal function is a significant regulator of adiponectin when categorized by CKD stage, whereas hypoadiponectinemia is a predictor of CVD, including recurrent IHD. (Am J Cardiol 2006;98:1603–1608)

Adiponectin is a key molecule in the metabolic syndrome[1–3] and is importantly involved in cardiovascular disease (CVD).[2,4] Recent data have suggest that hypoadiponectinemia is a novel putative CVD risk factor even in patients with mild to moderate renal failure,[5] although it has not been fully elucidated as to whether adiponectin concentration could be used as a predictive marker of CVD, separate from its increase by renal dysfunction.[6,7] Further, the clinical importance of adiponectin as a predictor of recurrent ischemic heart disease (IHD) has not been addressed. Thus, to examine whether renal function affects adiponectin concentration biologically, we carried out cross-sectional and longitudinal studies and evaluated its predictive power for CVD. Further, because different adiponectin isoforms have been reported to have certain clinical implications,[8] we examined the relative abundance of adiponectin isoforms in patients with severe chronic kidney disease (CKD).

Methods

Subjects: In total, 150 subjects were selected from among patients who were admitted and underwent medical investigation at the National Cardiovascular Center in Osaka, Japan. IHD was defined as ≥75% organic stenosis of ≥1 major coronary artery confirmed by coronary angiography or a history of myocardial infarction. All subjects with IHD in this study had undergone percutaneous transluminal coronary angioplasty before the initial assessment. Diabetes mellitus was defined according to criteria of the American Diabetes Association.[9] Hypertension was defined as a systolic blood pressure ≥140 mm Hg and/or a diastolic blood pressure ≥90 mm Hg on repeated measurements or use of antihypertensive treatment. Smoking was defined as current smoking or a history of habitual smoking. Subjects with acute coronary syndrome, cardiogenic shock, hemodialysis treatment, nephrotic syndrome, overt congestive heart failure, valvular heart disease, or atrial fibrillation were excluded. Further, no subjects receiving erythropoietin or steroid therapy were included in this study. All procedures in the present study were carried out in accordance with institutional and national ethical guidelines for human stud-

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ies. All subjects enrolled in this study were Japanese and gave informed consent to participate in the study.

Laboratory measurements: After subjects fasted overnight, blood pressure was measured by well-trained physicians with a mercury column sphygmomanometer, and venous blood was drawn from all subjects. Height and body weight were measured, and body mass index was calculated. Plasma samples for subsequent assay were stored at −80°C. Plasma concentration of adiponectin was determined by a sandwich enzyme-linked immunosorbent assay system (Adiponectin Enzyme-Linked Immunosorbent Assay Kit, Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) as previously reported.[1] Urine samples were collected for 3 days and averaged to evaluate creatinine clearance. The parameters hemoglobin, hematocrit, total cholesterol, triglycerides, and high-density lipoprotein cholesterol were also determined.

Follow-up study: After the initial assessment, patients were monitored for 31.9 ± 1.5 months. During follow-up, CVD events were accurately recorded. CVD events of interest in this study were myocardial infarction and angina pectoris confirmed by electrocardiographic changes; coronary angiographic and/or myocardial scintigraphic findings; stroke and transient cerebral ischemia confirmed by clinical symptoms; and computed tomographic, magnetic resonance angiographic, and/or cerebrovascular angiographic findings. IHD events included angina pectoris and myocardial infarction. In addition, restenosis of a lesion that had previously been subjected to percutaneous transluminal angioplasty at the initial assessment was not included as a CVD or IHD event for this analysis. Cause of death was classified as CVD, if there was sudden death from CVD, by an independent review panel of physicians who were unaware of echocardiographic and clinical findings. Events that were more equivocal, such as unrecognized myocardial infarction, angina pectoris, and transient cerebral ischemia, were not included as CVD for this analysis. For patients who developed multiple nonfatal episodes of CVD, the analysis included only the first event.

Because kidney transplantation affects adiponectin concentration,[10] patients receiving kidney transplants throughout follow-up were excluded from this study.

Analysis of oligometric state of adiponectin in plasma: Seven men (65.7 ± 3.2 years of age; body mass index 22.0 ± 1.5 kg/m^2) who were hospitalized in the National Cardiovascular Center were enrolled in this study. Methods of blood sampling and exclusion criteria of this study were identical to those previously described. Further, in this study, no subjects with CVD at the initial assessment were included and did not receive renal replacement therapy throughout follow-up. The relative isoform distribution of adiponectin was determined as previously reported.[8]

Statistical analysis: Data are expressed as mean ± SE. Levels of adiponectin were log-transformed for linear regression models, and relations between adiponectin and various parameters were assessed using univariate linear regression analysis and the Pearson correlation coefficient. Levels of adiponectin were assessed according to baseline creatinine clearance. Patients were categorized into 4 groups according to category of CKD as defined by Kidney Disease Outcomes Quality Initiative (K/DOQI) clinical practice guidelines for CKD as approximated by creatinine clearance (≥90, 60 to 90, 30 to 60, <30 ml/min),[11] i.e., groups 1 to 5, and then the significance of differences between groups was evaluated using 1-way of analysis of variance with the Dunnett multiple comparison post test. Multiple regression models were used to assess the relation between adiponectin levels and CKD groups after adjustment for age, gender, smoking habits, body mass index, hypertension, diabetes, total cholesterol, triglycerides, high-density lipoprotein cholesterol, and hemoglobin.

All subjects were categorized into 2 groups according to median baseline adiponectin level by each gender. Event-

Table 1
Clinical characteristics of total subjects (n = 150)

Variable	
Age (yrs)	67.7 ± 0.8
Men/women	102/48
Body mass index (kg/m^2)	23.5 ± 0.3
Smoker	72.7%
Previous IHD	43.3%
Diabetes mellitus	43.3%
Hypertension	87.3%
Adiponectin (μg/ml)*	4.8 (3.0, 9.8)
Hemoglobin (g/L)	128 ± 2
Hematocrit	0.39 ± 0.01
Systolic blood pressure (mm Hg)	139 ± 2
Diastolic blood pressure (mm Hg)	74 ± 1
Total cholesterol (mg/dl, mmol/L)	191 ± 3, 4.93 ± 0.07
Triglycerides (mg/dl, mmol/L)	112 ± 4, 1.27 ± 0.05
HDL cholesterol (mg/dl, mmol/L)	44.9 ± 1.2, 1.16 ± 0.03
Creatinine clearance (ml/min)	66.3 ± 3.5
CKD stage	
1	24.7%
2	32.0%
3	23.3%
4–5	20.0%

Values are means ± SEs.
* Values are medians (first and third quartiles).
HDL = high-density lipoprotein.

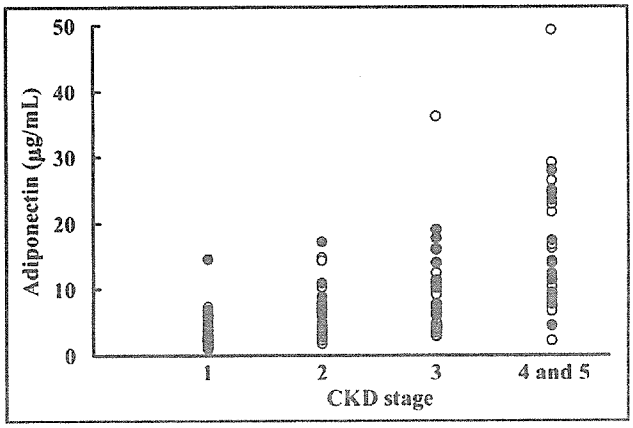

Figure 1. Baseline levels of adiponectin according to CKD stages in men (n = 102) *(gray circles)* and women (n = 48) *(white circles)*.

Table 2
Chronic kidney disease categories as a predictor of log-transformed adiponectin levels in the study

CKD Categories	Crude Model		Adjusted Model‡	
	Adiponectin* (μg/ml)	p Value	Mean Adiponectin† (μg/ml)	p Value
1	3.1 (2.2, 4.7)		5.4 ± 1.1	
2	4.0 (2.8, 6.8)	<0.05	5.7 ± 1.0	NS
3	6.4 (4.4, 11.1)	<0.01	8.9 ± 1.0	<0.01
4–5	11.9 (8.2, 23.0)	<0.01	12.1 ± 1.6	<0.01

* Values are presented as median (first and third quartiles).
† Values are presented as mean ± SE.
‡ Adjusted by age, gender, body mass index, smoker, diabetes, hypertension, previous IHD, triglycerides, high-density lipoprotein cholesterol, and hemoglobin.

Table 3
Clinical characteristics of subjects in follow-up study

Variable	Group 1* (n = 76)	Group 2† (n = 74)
Age (yrs)	69.4 ± 1.1	65.9 ± 1.1§
Men/women	51/25	51/23
Body mass index (kg/m^2)	22.1 ± 0.5	24.9 ± 0.5‖
Smoker	71.1%	74.3%
Previous IHD	43.4%	43.2%
Diabetes mellitus	42.1%	44.6%
Hypertension	86.8%	87.8%
Adiponectin (μg/ml)‡	6.9 (4.7, 9.5)	2.4 (1.8, 3.0)‖
Hemoglobin (g/L)	117 ± 2	139 ± 2‖
Hematocrit	0.36 ± 0.01	0.42 ± 0.01‖
Systolic blood pressure (mm Hg)	135 ± 2	142 ± 2§
Diastolic blood pressure (mm Hg)	74 ± 1	74 ± 1
Total cholesterol (mg/dl, mmol/L)	191 ± 4, 4.95 ± 0.10	190 ± 4, 4.91 ± 0.10
Triglycerides (mg/dl, mmol/L)	105 ± 5, 1.18 ± 0.06	121 ± 5§, 1.37 ± 0.06§
HDL cholesterol (mg/dl, mmol/L)	47.2 ± 1.2, 1.22 ± 0.04	42.5 ± 1.2§, 1.10 ± 0.04§
Creatinine clearance (mL/min)	42.2 ± 4.6	89.4 ± 4.7‖
No. of CVD events	12	19

Values are presented as mean ± SE.
* Adiponectin ≥4.39 μg/ml in men, ≥6.84 μg/ml in women.
† Adiponectin <4.39 μg/ml in men, <6.84 μg/ml in women.
‡ Values are presented as median (first and third quartiles).
§ $p < 0.05$; ‖ $p < 0.01$ versus group 1.
Abbreviation as in Table 1.

free survival analysis was performed with the Kaplan-Meier method to plot the cumulative incidence of CVD, and groups were compared by the Mantel log-rank test. Cox proportional hazard analysis was used to examine the association between variables and the cumulative incidence of CVD. Hazard ratios and their 95% confidence intervals were calculated using estimated regression coefficients and their SEs in Cox regression analysis.

In adiponectin isoform, statistical significance was tested using unpaired *t* tests. A p value <0.05 was considered statistically significant. All calculations were performed using JMP 4.0 (SAS Institute, Cary, North Carolina).

Results

Relation between adiponectin and renal dysfunction: Clinical and biochemical characteristics of study subjects are presented in Table 1. Adiponectin was significantly correlated with age ($r = 0.31$, $p < 0.01$), body mass index ($r = -0.33$, $p < 0.01$), hemoglobin ($r = -0.63$, $p < 0.01$), hematocrit ($r = -0.60$, $p < 0.01$), systolic blood pressure ($r = -0.30$, $p < 0.01$), triglycerides ($r = -0.35$, $p < 0.01$), high-density lipoprotein cholesterol ($r = 0.29$, $p < 0.01$), and creatinine clearance ($r = -0.65$, $p < 0.01$), and was decreased in men (7.1 ± 0.7 vs 9.1 ± 1.0 μg/ml, $p < 0.01$) and smokers (7.4 ± 0.7 vs 8.6 ± 1.1 μg/ml, $p < 0.05$). Figure 1 shows plots of patient adiponectin levels according to CKD categories at enrollment. Table 2 lists cross-sectional data regarding baseline CKD categories as predictors of log-transformed adiponectin levels. In crude and adjusted models, increasing categories of CKD were significant predictors of adiponectin levels (p for trend <0.01 for the 2 comparisons).

Plasma adiponectin concentration and CVD events: During follow-up, 31 patients (5 women) developed CVD. There were 2 with myocardial infarction, 13 with angina pectoris, 9 with cerebral infarction, and 7 with transient cerebral ischemia. Adiponectin was significantly lower in subjects

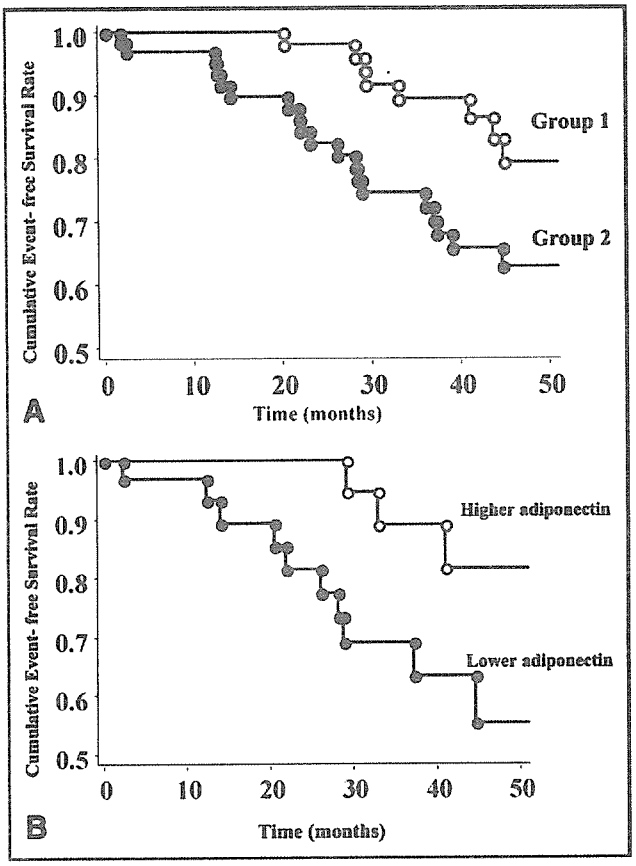

Figure 2. Kaplan-Meier survival curves for *(A)* CVD events in all subjects (n = 150, log-rank chi-square 4.88, p <0.03) and *(B)* IHD events in the subgroup with previous IHD (n = 65, log-rank chi-square 3.96, p <0.05). Subjects were stratified into 2 groups according to median adiponectin concentration by each gender.

who developed CVD events during follow-up (5.0 ± 1.3 vs 8.4 ± 0.7 μg/ml, p <0.02). Because of the gender-specific difference in adiponectin levels,[12] different median values for men and women were used to separate the higher group from the lower group of adiponectin (4.39 μg/ml in men, 6.84 μg/ml in women). Their clinical characteristics are listed in Table 3. Age, adiponectin, body mass index, hemoglobin, hematocrit, triglycerides, high-density lipoprotein cholesterol, and creatinine clearance were significantly different between the higher adiponectin group (group 1) and lower adiponectin group (group 2). Figure 2 shows the life-table analysis of CVD events throughout the follow-up period using the Kaplan-Meier method. These curves illustrate significantly poorer event-free survival of group 2. Results of Cox proportional hazard models are presented in Table 4, and the predictive value of adiponectin for CVD events in crude, CKD stage-adjusted, and risk factor-adjusted models are presented. Because previous IHD and smoking habit were also associated with a higher risk of CVD events, we selected these parameters as risk factors. Even after adjusting for CKD stages, previous IHD, and smoking habit, decreased adiponectin levels were a significant predictor of CVD in all models. The risk factor-adjusted Cox regression showed that an increase in adiponectin per 1 μg/ml was associated with a decrease in the risk of CVD to 0.86.

When the analysis was restricted to subjects with previous IHD (n = 65), 15 recurrent IHD events occurred during follow-up. Even in these subjects, adiponectin showed a tendency to be decreased in subjects who developed recurrent IHD events during follow-up (4.3 ± 1.9 vs 7.8 ± 1.1 μg/ml, p = 0.06). Different median values of adiponectin for men and women were also used to separate the higher group from the lower group (4.45 μg/ml in men, 4.49 μg/ml in women), and life-table analysis of recurrent IHD events throughout follow-up was performed using the Kaplan-Meier method (Figure 2). These curves showed significantly lower event-free survival of the lower adiponectin group. In addition, in Cox regression analysis, lower adiponectin was associated with a 1.85-fold higher risk of recurrent IHD events (hazard ratio 1.85, 95% confidence interval 1.02 to 3.92, p <0.05).

Relative plasma adiponectin isoform levels in patients with severe CKD: The ratio of the 3 major isoforms of adiponectin was analyzed in 7 men with severe CKD (creatinine clearance 8.9 ± 2.1 ml/min), and 3 IHD events occurred during follow-up. Adiponectin concentration was significantly lower in the subjects who had IHD events during follow-up (9.1 ± 0.1 vs 19.2 ± 2.9 μg/ml, p <0.04). Averaged elution profiles of adiponectin forms in plasma (Figure 3) and the percentage of each form of adiponectin in total adiponectin (Figure 3) are shown. The percentage of the high-molecular-weight form in total adiponectin was significantly lower in subjects who had IHD than in those who did not (−16.9%, p <0.02; Figure 3), whereas those of the hexamer and trimer in total adiponectin were not significantly different between the 2 groups. In addition, in the subjects without IHD throughout the follow-up period, the percentage of high molecular weight was significantly higher than that of the hexamer and trimer (vs hexamer +18.9%, p <0.05, vs trimer +39.6%, p <0.01; Figure 3).

Discussion

The present study demonstrated that renal function categorized by CKD stage was independently associated with adiponectin concentration. However, low adiponectin was a predictor of CVD, separately from its increase induced by renal dysfunction. Further, even in patients with previous IHD, low adiponectin may be a predictor of recurrent IHD. In the relative distribution of adiponectin isoforms, the percentage of the high-molecular-weight form in patients with severe CKD without IHD throughout the follow-up period was significantly higher than that in those who developed IHD.

Previous data from smaller studies have suggested that adiponectin levels are related to renal function in subjects with hypertension,[13] end-stage renal failure treated with hemodialysis therapy,[14] and renal diseases (including nephrotic syndrome).[15] Further, another recent report has suggested that renal dysfunction estimated by urea nitrogen may be the cause of hyperadiponectinemia in the elderly.[6] The present study extended these observations for adiponectin among the CKD stages of renal dysfunction, and multiple linear regression analysis clearly

Table 4
Adiponectin as a predictor of cardiovascular disease events

Variables, Unit of Increase	Crude		Adjusted CKD Stage		Adjusted Model*	
	HR (95% CI)	p Value	HR (95% CI)	p Value	HR (95% CI)	p Value
Adiponectin, 1 μg/ml increase	0.88 (0.79–0.97)	0.004	0.84 (0.72–0.94)	0.001	0.86 (0.75–0.96)	0.004
Smoker, yes	1.88 (1.14–3.24)	0.045	1.86 (1.12–3.21)	0.056	1.65 (0.98–2.89)	0.166
Previous IHD	1.61 (1.11–2.38)	0.011	1.59 (1.10–2.36)	0.014	1.49 (1.09–2.75)	0.041

* Adjusted by previous IHD, smoking, and CKD stages.
CI = confidence interval; HR = hazard ratio.

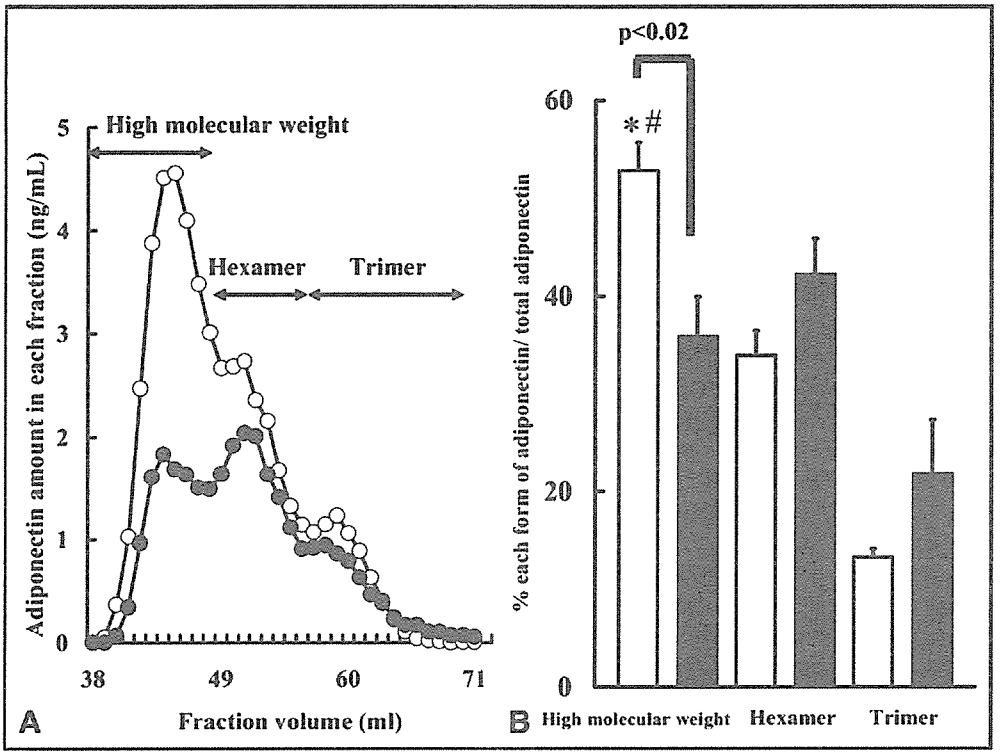

Figure 3. Oligometric state of adiponectin in human plasma from patients with severe CKD. *(A)* Averaged elution profiles of adiponectin in plasma from subjects without IHD (n = 4) *(white circles)* and those with IHD (n = 3) *(black circles)*. Plasma was fractionated by gel filtration chromatography, and the concentration of adiponectin in each 1-ml fraction was determined by enzyme-linked immunosorbent assay. *(B)* Percentage of each form of adiponectin in total adiponectin from subjects without IHD (n = 4) *(white bars)* and those with IHD (n = 3) *(black bars)*. Values are means ± SEs. *p <0.05 versus hexamer; #p <0.01 versus trimer.

showed that CKD stages were independently associated with adiponectin concentration. Thus, our results also confirm that the kidney is the target organ regulating adiponectin concentration.

In this study, we carried out a prospective study by categorizing subjects into 2 groups according to median adiponectin concentration. Our results were partly in accordance with previous reports associating a higher adiponectin concentration with a lower risk of CVD in men[16] and in subjects with end-stage renal failure[14] and mild and moderate renal failure.[5] The present study showed that increased adiponectin was significantly associated with low risk of CVD events, even after adjustment for CKD stages, previous IHD, and smoking. In addition, even when the analysis was restricted to subjects with previous IHD, lower adiponectin was linked to a higher rate of recurrent IHD events. These results suggest that lower adiponectin concentration may be a potential risk factor for IHD, including recurrent IHD.

Previous reports have shown that high-molecular-weight adiponectin specifically confers the vasoprotective activities of this protein.[8] Our results lead to the notion that increased adiponectin in renal dysfunction, especially the high-molecular-weight isoform, may exert a protective role against CVD. However, further investigation is required to examine this hypothesis.

In conclusion, although renal function is a significant regulator of adiponectin, hypoadiponectinemia is a predictor of CVD, including recurrent IHD, apart from its increase induced by renal dysfunction.

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Plasma Adiponectin Levels Are Associated With Coronary Lesion Complexity in Men With Coronary Artery Disease

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OBJECTIVES We sought to assess whether plasma adiponectin levels correlate with angiographic coronary lesion complexity in patients with coronary artery disease (CAD).

BACKGROUND Metabolic disorders, including diabetes mellitus and metabolic syndrome, are important risk factors for acute cardiovascular events, and adiponectin is a key molecule of metabolic disorders, with anti-atherogenic properties. Low plasma adiponectin levels are associated with CAD and future incidence of myocardial infarction. The involvement of adiponectin in coronary plaque vulnerability, which may be reflected by angiographic complex lesions, remains to be elucidated.

METHODS We measured plasma adiponectin levels in 207 men (152 with stable CAD and 55 with acute coronary syndromes [ACS]). Coronary lesions were classified as of simple or complex appearance.

RESULTS Plasma adiponectin levels were significantly lower in stable CAD patients with complex coronary lesions (n = 60) than in those with simple lesions (n = 92) (4.14 [range 2.95 to 6.02] vs. 5.27 [range 3.67 to 8.12] μg/ml, p = 0.006). Multiple logistic regression analysis demonstrated that adiponectin level was independently associated with complex lesions (odds ratio 0.514, 95% confidence interval 0.278 to 0.951; p = 0.034). Polytomous logistic regression revealed that adiponectin correlated independently with both single and multiple complex lesions. Among patients with ACS, who had lower adiponectin levels than stable CAD patients, those with multiple complex lesions had significantly lower adiponectin than those with a single complex lesion (3.26 [range 2.26 to 4.46] vs. 4.21 [range 3.36 to 5.41] μg/ml, p = 0.032).

CONCLUSIONS Plasma adiponectin levels are significantly associated with coronary lesion complexity in men with CAD. Low adiponectin levels may contribute to coronary plaque vulnerability. (J Am Coll Cardiol 2006;48:1155–62) © 2006 by the American College of Cardiology Foundation

The incidence of diabetes mellitus, a major and important risk factor for cardiovascular events including acute coronary syndromes (ACS), is increasing worldwide (1). Similarly, the metabolic syndrome, a clustering of cardiovascular disease risk factors characterized by abdominal obesity, insulin resistance, dyslipidemia, and hypertension, is associated with increased cardiovascular morbidity and mortality (2). There is increasing evidence that adiponectin, an adipocyte-derived plasma protein, plays an important role in the development of diabetes mellitus and metabolic syndrome, with anti-diabetic and anti-atherogenic properties (3). Plasma levels of adiponectin are significantly decreased in obesity (4), in type 2 diabetes (5), and in patients with coronary artery disease (CAD) (6). In addition, we recently reported a significant association between plasma adiponectin levels and atherosclerotic burden (7).

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Hypoadiponectinemia is considered an independent risk factor for CAD (8), and a recent study demonstrated that plasma adiponectin levels in patients with ACS were significantly lower than those in patients with stable CAD (9). Moreover, it has been shown (10) that lower levels of plasma adiponectin are associated with increased risk of future myocardial infarction (MI). Thus, low adiponectin may contribute to the development of atherosclerosis and acute vascular complications including ACS.

The vulnerability of coronary plaques is implicated in the pathogenesis of ACS (11). Vulnerable atheromatous plaques lead to coronary plaque disruption with superimposed thrombosis (12), which is often manifested as angiographically complex lesions (13). The presence of complex coronary lesions is associated with acute coronary events

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