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Adiponectin inhibits the binding of low-density lipoprotein to biglycan, a vascular proteoglycan ☆

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Abstract

The aim of this study was to test the possibility that adiponectin has an antiatherogenic effect through the inhibition of LDL binding to proteoglycans, an initial event in atherogenesis. Both full-length and globular adiponectin inhibited LDL binding in a dose-dependent manner. Both types of adiponectin bound to biglycan in a dose-dependent manner. Immunoprecipitation and immunoblotting analysis showed interaction of full-length adiponectin with LDL. Pretreatment of biglycan with globular adiponectin prior to LDL addition diminished the inhibitory effect, while pretreatment with full-length adiponectin retained the effect. This is a new antiatherogenic property that appears independent of the receptor-mediated hormonal action of adiponectin.

Keywords: Adiponectin; Biglycan; Extracellular matrices; Proteoglycan; LDL retention; Apolipoprotein B; Full-length; Globular; Atherosclerosis; Solid-phase plate assays

There is a growing body of evidence that supports the hypothesis that subendothelial retention of apolipoprotein B100-containing lipoproteins is the initial event in atherosclerosis [1]. The extracellular matrices in the subendothelial space, in particular proteoglycans, are considered to play a crucial role in the retention of lipoproteins [2,3]. It has been shown that the binding of lipoproteins to biglycan, a vascular proteoglycan, is modulated by several factors, such as defensin [4], lipoprotein lipase [5], phospholipase A_2 [6], and apolipoprotein C-III [7]. It is possible that these modulators can modify the initiation and progression of atherosclerosis. On the other hand, adipocyte-derived adiponectin has been recognized as an antiatherogenic factor and was shown to be significantly lower in individuals with coronary artery disease than in age- and body mass index-adjusted control subjects [8,9]. Investigators reported in several in vitro studies that adiponectin suppressed expression of adhesion molecules in endothelial cells [8], cholesterylester accumulation in macrophages [10], and proliferation of smooth muscle cells [11], while in vivo studies showed antiatherogenic effects in adiponectin-deficient mice [12,13] or apolipoprotein E-deficient mice [14] by adenovirus-mediated supplement of adiponectin [12].

An early report showed that adiponectin infiltrated rapidly into the subendothelial space of the vascular wall when the endothelial barrier was injured with balloon angioplasty [15]. Adiponectin was also shown to bind to vascular matrix proteins, such as collagen types I, III, and V [15]. Thus, we investigated the possibility that adiponectin has an antiatherogenic effect through the inhibition of LDL binding to proteoglycans.

☆ *Abbreviations:* LDL, low-density lipoprotein; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HBS, Hepes-buffered saline.

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Materials and methods

Lipoprotein preparation. LDL was purchased from Athens Research and Technology (Athens, GA, USA) or prepared from plasma obtained from normolipidemic volunteers who had fasted for 12–14 h. Blood was drawn from antecubital veins into tubes containing 6.7 mM EDTA. LDL was isolated by sequential ultracentrifugation at a density of 1.019–1.063 g/ml according to the method of Havel et al. [16]. LDL collected was dialyzed, sterilized, and stored at 4 °C until use.

Plate-assay analysis with biglycan. The solid-phase assay of LDL binding to biglycan with or without adiponectin was performed as described by Skalen et al. [3]. For the preincubation study, LDL was added after overnight incubation of biglycan with adiponectin, followed by washing with a washing buffer. The plate assay of adiponectin to biglycan was carried out as follows. Maxisorp immunoplates (Nalge Nunc International, Rochester, NY, USA) were coated with 10 μg/ml of biglycan (Sigma Chemical, St. Louis, MO, USA) in HBS buffer (20 mM Hepes, 150 mM NaCl, pH 7.4) overnight at room temperature and blocked with HBS buffer with 1% bovine serum albumin for 1 h at room temperature. Plates were incubated with various concentrations of adiponectin in HBS buffer overnight at 4 °C. One hundred microliters of anti-human adiponectin antibody was added and the plates were again incubated overnight at 4 °C. They were then incubated with the secondary antibody (mouse anti-rabbit IgG-HRP; Amersham Biosciences, Piscataway, NJ, USA) at a dilution of 1:5000 for 1.5 h at room temperature. Finally, 100 μl of Turbo TMB-ELISA (Pierce, Rockford, IL, USA) substrate was added and incubated for 5 min at 37 °C. The reaction was stopped with an equal volume of 2 M H_2SO_4 and the samples were measured at 450 nm in a spectrophotometer.

Gel mobility shift analysis. The interaction between adiponectin and LDL was investigated using a gel mobility shift analysis. Increasing concentrations of adiponectin were incubated with 7.5 μg of LDL overnight at 4 °C. Electrophoresis was performed at 90 V for 1 h in 60 mM barbital buffer using the TITAN GEL Lipoprotein Electrophoresis System (Helena Laboratories, Saitama, Japan). Proteins were transferred to 0.2 μm nitrocellulose (Trans-Blot; Bio-Rad, Hercules, CA, USA). Blots were probed with a HRP-conjugated polyclonal antibody against human apoB (The Binding Site, Birmingham, UK) used at a dilution of 1:750 in 10 mM phosphate-buffered saline with 0.1% Tween 20. The signal was detected by a chemiluminescent reaction (ECL Plus; Amersham).

Immunoprecipitation and immunoblotting. Control IgG, goat anti-human apolipoprotein B antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or mouse anti-human adiponectin antibody (Chemicon International, Temecula, CA, USA) was preabsorbed on Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) for 3 h at 4 °C. Mixture samples of adiponectin and LDL were incubated with agarose conjugate overnight at 4 °C, followed by control IgG-agarose conjugate for 3 h at 4 °C, and finally incubated with the specific antibody–agarose conjugate overnight at 4 °C with mixing by end over end rotation. The immunocomplexes on the agarose conjugate were washed six times with washing buffer and separated in 10% (Bio-Rad) or 2–15% SDS–PAGE gels (Daiichi Pure Chemicals, Tokyo, Japan) under reducing conditions. Proteins were transferred to 0.2 μm nitrocellulose (Bio-Rad). Blots were probed with the following primary antibodies: an anti-human apolipoprotein B antibody used at a dilution of 1:500 and an anti-human adiponectin antibody used at a dilution of 1:5000. Sheep anti-mouse IgG-HRP (Amersham) and donkey anti-goat IgG-HRP (Promega, Madison WI, USA) were used at 1:5000. The signal was detected by a chemiluminescent reaction (Amersham).

Statistical analysis. Non-parametric analysis of data was performed with Statview version 5 using one-way analysis of variance followed by comparisons using Fischer's PLSD method. A value of $p < 0.05$ denoted the presence of a statistically significant difference.

Results

Fig. 1 shows the effect of adiponectin on LDL binding to biglycan in solid-phase plate assays. Full-length (Fig. 1A) and globular (Fig. 1B) adiponectin inhibited LDL binding to biglycan in a dose-dependent manner. Bound LDL in the presence of 20 μg/ml of adiponectin was significantly lower than bound LDL without adiponectin (full-length: 0.137 ± 0.02 vs. 0.237 ± 0.02 arbitrary units, $n = 6$, $p < 0.005$; globular: 0.114 ± 0.037 vs. 0.254 ± 0.049, $n = 6$, $p < 0.001$). Fig. 2 demonstrates both types of adiponectin bound to biglycan. However, globular adiponectin (Fig. 2B) seemed to show low affinity binding compared with full-length adiponectin (Fig. 2A). Full-length adiponectin was detected in immunoprecipitates obtained with anti-apolipoprotein B antibody from a mixture of adiponectin and LDL

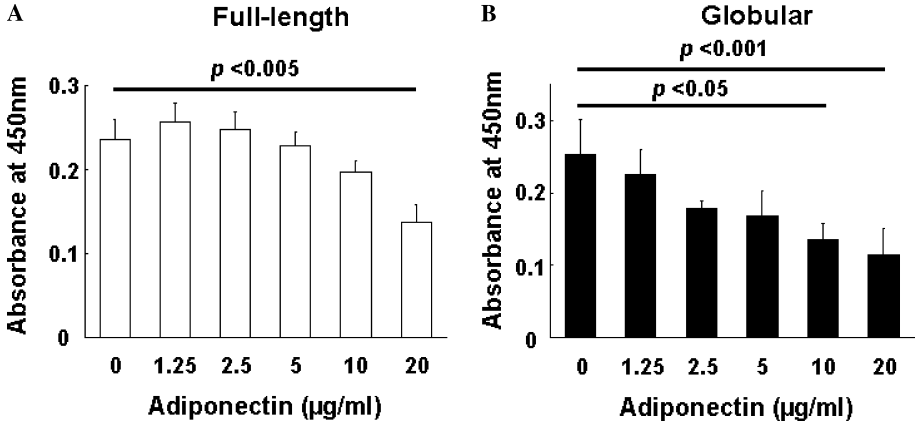

Fig. 1. Effects of full-length (A) and globular (B) adiponectin on LDL binding to biglycan. 96-well plates were coated with 10 μg/ml of biglycan overnight at room temperature and blocked with 1% bovine serum albumin for 1 h at room temperature. The plates were incubated with both LDL and various concentrations of adiponectin overnight at 4 °C. Anti-human apolipoprotein B antibody was added and incubated overnight at 4 °C. Finally, ELISA substrate was added and incubated for 5 min at 37 °C. The reaction was stopped with an equal volume of 2 M H_2SO_4 and measured at 450 nm in a spectrophotometer. Values are means + SE ($n = 6$).

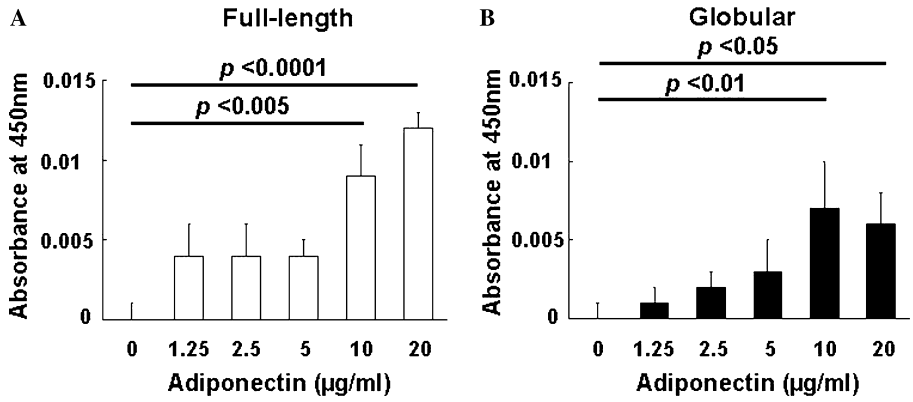

Fig. 2. Binding of full-length (A) and globular (B) adiponectin to biglycan with solid-phase plate assay. The plate assay was performed as described in Materials and methods. Values are means + SE ($n = 6$).

(Fig. 3A). The reverse experiment, where adiponectin was first immunoprecipitated and the immunoprecipitates were then probed with anti-apolipoprotein B antibody, yielded an apolipoprotein B band (Fig. 3B). Interestingly, apolipoprotein B was detected in immunoprecipitates obtained with anti-adiponectin antibody from a LDL sample without adiponectin; a result that suggests adiponectin is associated with LDL in blood. The mobility of LDL incubated with both types of adiponectin was increased in a dose-dependent manner, though full-length adiponectin (Fig. 3C) showed much less mobility shift than globular adiponectin (Fig. 3D). These results suggested that adiponectin bound to LDL and induced a greater negative charge. Treatment of biglycan with full-length adiponectin prior to LDL addition showed a similar inhibitory effect (Fig. 4A), while globular adiponectin showed an inhibitory effect only when added simultaneously with LDL (Fig. 4B).

Discussion

Adiponectin is an adipocyte-derived factor that has been proposed to play an important role in the regulation of energy homeostasis and insulin sensitivity [13,17]. Furthermore, adiponectin has antiatherogenic properties with epidemiological studies showing that plasma concentrations of adiponectin are decreased in obese individuals [18], type 2 diabetes mellitus [19], essential hypertension [20], and coronary heart disease [8,9]. Stud-

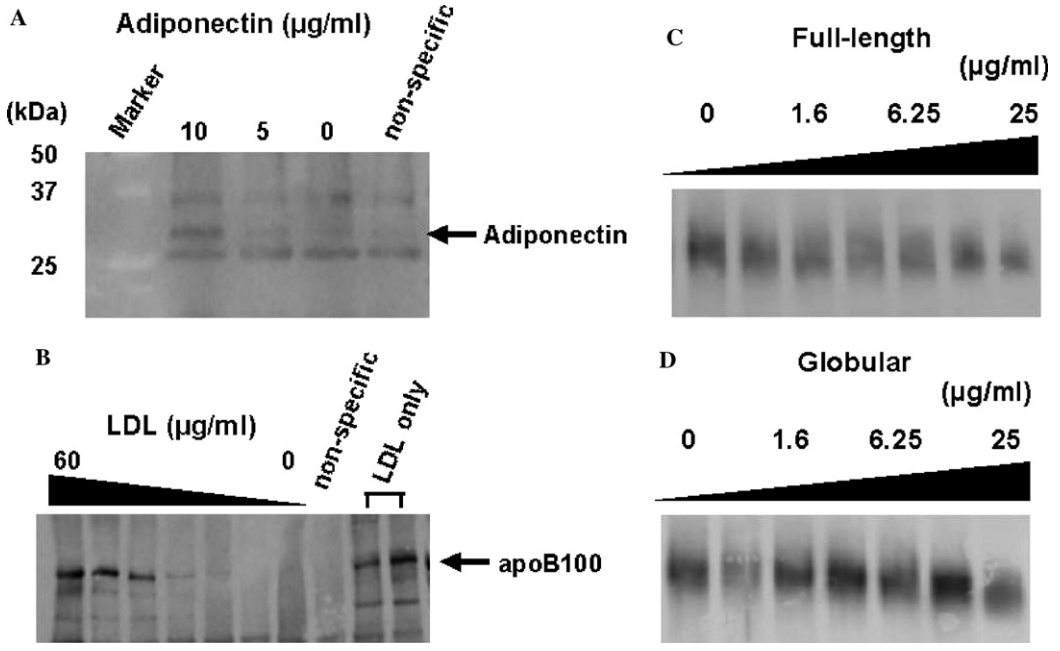

Fig. 3. Immunoprecipitation and gel mobility shift analysis of association between adiponectin and LDL. (A) The mixture samples of full-length adiponectin and LDL were immunoprecipitated with an anti-human apolipoprotein B antibody. The immunoprecipitates were probed with anti-adiponectin antibody. (B) The reverse experiment to (A). Immunoprecipitation using control IgG was labeled non-specific. The mobility of mixture samples of LDL and full-length (C) or globular (D) adiponectin were investigated using agarose gel electrophoresis.

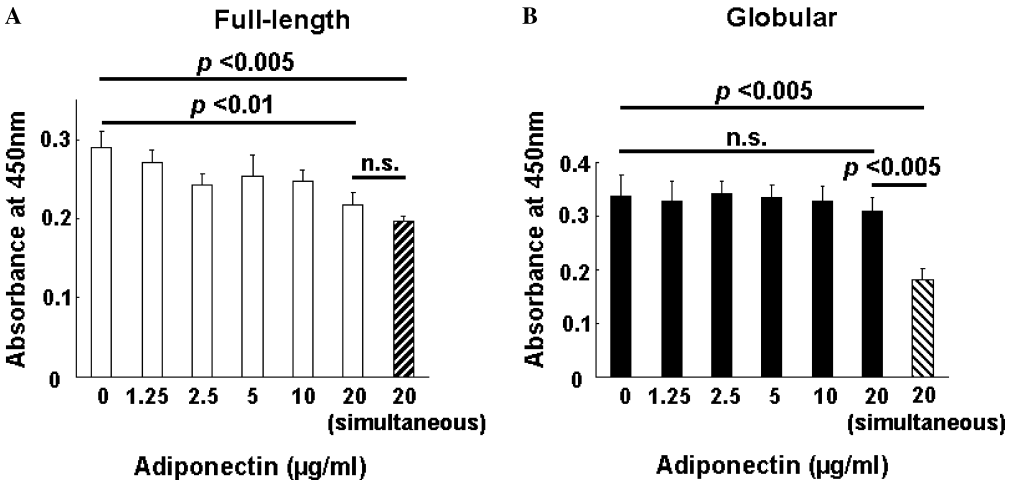

Fig. 4. Effects of preincubation with full-length (A) and globular (B) adiponectin on LDL binding to biglycan. The plate assay was performed as described for Fig. 1 except that incubation of biglycan with adiponectin was followed by washing prior to addition of LDL. Values are means + SE ($n = 6$).

ies in vivo have reported that neointimal thickening after artery injury was increased in adiponectin-deficient mice [12,13] and was attenuated by adenovirus-mediated supplement of adiponectin [12]. Furthermore, adenovirus-induced increases in plasma adiponectin reduced atherosclerosis in apolipoprotein E-deficient mice [14]. Several studies have reported possible underlying mechanisms for the antiatherogenic effects of adiponectin; adiponectin suppressed expression of adhesion molecules in endothelial cells [8], cholesterylester accumulation in macrophages [10], and proliferation of smooth muscle cells [11]. However, no report has so far shown the effects of adiponectin on binding of apolipoprotein B100-containing lipoproteins to vascular proteoglycans as an initial event in atherogenesis. According to the response to retention hypothesis of atherogenesis, two processes thought to be central to the development of atherosclerosis are the retention of lipoproteins by binding to proteoglycans in the arterial wall and subsequent modification of these retained lipoproteins by processes such as oxidation [1]. Skalen et al. [3] reported that mice expressing proteoglycan-binding-defective LDL showed the same binding ability to the LDL receptor. The cholesterol level of the proteoglycan-binding-defective LDL mice was the same as normal control mice, but they developed significantly less atherosclerosis than mice expressing the wild-type control. Therefore, it is possible that inhibition of LDL binding to biglycan could suppress initiation and development of atherosclerosis. In the present study, we showed adiponectin inhibited LDL binding to biglycan in a dose-dependent manner by plate-assay analysis. This effect of adiponectin on LDL binding occurred at a concentration of 10–20 μg/ml, well within the range found in human plasma [18,19]. The mechanism for this inhibitory effect remains unclear. Okamoto et al. [15] reported that adiponectin adhered rapidly to the subendothelial space of injured vascular walls and suggested that adiponectin was a kind of matrix protein because of its high sequence homology to collagens VIII and X. They also showed that adiponectin bound to vascular matrix proteins, such as collagen types I, III, and V. On the other hand, several groups have reported that proteoglycan–lipoprotein binding involves an interaction between negative charges on the glycosaminoglycan chains of proteoglycans and positively charged lysine and arginine residues on 'site B' (residues 3359–3369) of apolipoprotein B [21,22] or E [23,24]. Our results showed both types of adiponectin bound to biglycan and to LDL. In addition, increasing concentrations of adiponectin added a greater negative charge to LDL. These results suggest that adiponectin competitively inhibits LDL binding to biglycan either through masking the binding sites of apolipoprotein B and/or through weakening the electric charge difference between apolipoprotein B and biglycan.

The effect of adiponectin on oxidized LDL binding to biglycans was not investigated in this study. It is usually believed that oxidation occurs only after native LDL binds to extracellular matrices and is isolated from antioxidants in plasma [3,25–27]. Kaplan and Aviram [28] showed that mildly oxidized LDL demonstrated increased interaction with the extracellular matrix, while the binding of extensively oxidized LDL was decreased. On delipidated apolipoprotein B, there are eight specific regions of clustered basic amino acids that might bind to the negatively charged biglycan. Therefore, it is possible that both the binding capacity of oxidized LDL and the inhibitory effect of adiponectin depend on the extent of oxidation of LDL.

In our study, the binding ability of globular adiponectin to biglycan seemed to be lower than that of full-length adiponectin. In addition, globular adiponectin only showed an inhibitory effect when added simultaneously

with LDL to biglycan. On the other hand, globular adiponectin had a greater effect of adding a negative charge to LDL than full-length adiponectin. These suggested that the collagen-like domain of adiponectin might be important for binding to biglycan, while the globular domain is important for binding to LDL. Globular adiponectin concentration in the serum was shown to be very low, but reversed the resistance to insulin more than full-length adiponectin [29]. Both types of adiponectin will be good candidates for therapeutic agents for the protection of vascular walls but their different inhibitory effects on LDL binding should be taken into consideration.

Low plasma levels of adiponectin were commonly observed in many different metabolic disorders. A decreased ratio of adiponectin to LDL may cause a decrease in protection of vascular walls from subendothelial retention of atherogenic lipoproteins and, if accompanied by dysregulation of energy homeostasis and insulin sensitivity under hypoadiponectinemia, can lead to accelerated progression of atherosclerosis.

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