Adiponectin Acts as an Endogenous Antithrombotic Factor

Hisashi Kato, Hirokazu Kashiwagi, Masamichi Shiraga, Seiji Tadokoro, Tsuyoshi Kamae, Hidetoshi Ujiie, Shigenori Honda, Shigeki Miyata, Yoshinobu Ijiri, Junichiro Yamamoto, Norikazu Maeda, Tohru Funahashi, Yoshiyuki Kurata, Iichiro Shimomura, Yoshiaki Tomiyama, Yuzuru Kanakura

Objective—Obesity is a common risk factor in insulin resistance and cardiovascular diseases. Although hypoadiponectinemia is associated with obesity-related metabolic and vascular diseases, the role of adiponectin in thrombosis remains elusive.

Methods and Results—We investigated platelet thrombus formation in adiponectin knockout (APN-KO) male mice (8 to 12 weeks old) fed on a normal diet. There was no significant difference in platelet counts or coagulation parameters between wild-type (WT) and APN-KO mice. However, APN-KO mice showed an accelerated thrombus formation on carotid arterial injury with a He-Ne laser (total thrombus volume: 13.36±4.25×10³ arbitrary units for APN-KO and 6.74±2.87×10³ arbitrary units for WT; n=10; P<0.01). Adenovirus-mediated supplementation of adiponectin attenuated the enhanced thrombus formation. In vitro thrombus formation on a type I collagen at a shear rate of 250 s⁻¹, as well as platelet aggregation induced by low concentrations of agonists, was enhanced in APN-KO mice, and recombinant adiponectin inhibited the enhanced platelet aggregation. In WT mice, adenovirus-mediated overexpression of adiponectin additionally attenuated thrombus formation.

Conclusion—Adiponectin deficiency leads to enhanced thrombus formation and platelet aggregation. The present study reveals a new role of adiponectin as an endogenous antithrombotic factor. (Arterioscler Thromb Vasc Biol. 2006;26:224-230.)

Key Words: acute coronary syndromes ■ obesity ■ platelets ■ thrombosis

Obesity is associated with insulin resistance, accelerated atherosclerosis, and cardiovascular diseases.¹,² Recent studies have revealed that adipose tissue is not only a passive reservoir for energy storage but also produces and secretes a variety of bioactive molecules, known as adipokines, including tumor necrosis factor (TNF) α, leptin, resistin, and plasminogen activator inhibitor type-1.²⁻⁴ Dysregulated production of adipokines participates in the development of obesity-related metabolic and vascular diseases.²⁻⁴

Adiponectin is an adipokine identified in the human adipose tissue cDNA library, and Acrp30/AdipoQ is the mouse counterpart of adiponectin (reviewed in reference⁵). Adiponectin, of which mRNA is exclusively expressed in adipose tissue, is a protein of 244 amino acids consisting of 2 structurally distinct domains, an N-terminal collagen-like domain and a C-terminal complement C1q-like globular domain. Adiponectin is abundantly present in plasma (5 to 30 μg/mL), and its plasma concentration is inversely related to the body mass index.³ Plasma adiponectin levels decrease in obesity, type 2 diabetes, and patients with coronary artery disease (CAD).³⁻⁹ Indeed, adiponectin (APN) knockout (KO) mice showed severe diet–induced insulin resistance.¹⁰ In cultured cells, we have demonstrated that human recombinant adiponectin inhibited the expression of adhesion molecules on endothelial cells, the transformation of macrophages to foam cells, and TNF-α production from macrophages.⁵,¹¹ Furthermore, APN-KO mice showed severe neointimal thickening in mechanically injured arteries.¹² Adenovirus-mediated supplementation of adiponectin attenuated the development of atherosclerosis in apolipoprotein E–deficient mice as well as postinjury neointimal thickening in APN-KO mice.¹²,¹³ These data suggest the antiatherogenic properties of adiponectin, and, hence, hypoadiponectinemia may be associated with a higher incidence of vascular diseases in obese subjects. Although it is also possible that an altered hemostatic balance may contribute to the pathogenesis of acute cardiovascular events in such patients, the roles of adiponectin in hemostasis and thrombosis remains elusive.
Here we have provided the first evidence that adiponectin affects thrombus formation, and, hence, hypoadiponectinemia may directly contribute to acute coronary syndrome. Our data indicate a new role of adiponectin as an antithrombotic factor.

Methods

Mice
APN-KO male mice (8 to 12 weeks old) were generated as described previously.10,12 We analyzed mice backcrossed to C57BL/6 for 5 generations.10,12

Preparation of Mouse Platelets and Measurement of Coagulation Parameters
Mouse platelet-rich plasma (PRP) was obtained as described previously.14 Coagulation parameters were measured by SRL Inc.

Platelet Aggregation Study, Adhesion Study, and Flow Cytometry
Platelet aggregation and platelet adhesion study was performed as described previously.14 Integrin αIIbβ3 activation and α-granule secretion of wild-type (WT) and APN-KO platelets were detected by phycoerythrin-conjugated JON/A monoclonal antibody (mAb), which binds specifically to mouse-activated αIIbβ3 (Emfret Analytics) and FITC-conjugated anti-P-selectin mAb (Becton Dickinson), respectively.14

Assessment of Atherosclerosis and Bleeding Time Measurement
Assessment of atherosclerosis was performed as described previously.15 The tail of anesthetized mice (nembutal 65 mg/kg; 8 to 12 weeks old) was transected 5 mm from the tip and then immersed in 0.9% isotonic saline at 37°C. The point until complete cessation of bleeding was defined as the bleeding time.

He-Ne Laser–Induced Thrombosis
The observation of real-time thrombus formation in the mouse carotid artery was performed as described previously.15 Anesthetized mice (nembutal 65 mg/kg) were placed onto a microscope stage, and the left carotid artery (450 to 500 μm in diameter) was gently exposed. Evans blue dye (20 mg/kg) was injected into the left femoral artery via an indwelt tube, and then the center of the exposed carotid artery was irradiated with a laser beam (200 μm in diameter at the focal plane) from a He-Ne laser (Model NEO-50MS; Nihon Kagaku Engineering Co, Ltd). Thrombus formation was recorded on a videotape through a microscope with an attached CCD camera for 10 minutes. The images were transferred to a computer every 4 s, and the thrombus size was analyzed using Image-J software (National Institutes of Health). We calculated thrombus size by multiplying each area value and its grayscale value together. We then regarded the total size values for an individual thrombus obtained every 4 s during a 10-minute observation period as the total thrombus volume and expressed them in arbitrary units.

Flow Chamber and Perfusion Studies
The real-time observation of mural thrombogenesis on a type I collagen-coated surface under a shear rate of 250 s⁻¹ was performed as described previously.16 Briefly, whole blood obtained from anesthetized mice was anticoagulated with argatroban, and then platelets in the whole blood were labeled by mepacrine. Type I collagen-coated glass cover slips were placed in a parallel plate flow chamber (rectangular type; flow path of 1.9-mm width, 31-mm length, and 0.1-mm height). The chamber was assembled and mounted on an epifluorescence microscope (Axiovert S100 inverted microscope; Carl Zeiss Inc) with the computer-controlled z-motor (Ludl Electronic Products Lts). Whole blood was aspirated through the chamber, and the entire platelet thrombus formation process was observed in real time and recorded with a video recorder.

Preparation of Adenovirus and Recombinant Adiponectin
Adenovirus producing the full-length mouse adiponectin was prepared as described previously.10 Plaque-forming units (1×10⁹) of adenovirus-adiponectin (Ad-APN) or adenovirus-β-galactosidase (Ad-βgal) were injected into the tail vein. Experiments were performed on the fifth day after viral injection. The plasma concentrations of adiponectin were measured by a sandwich ELISA. Mouse and human recombinant proteins of adiponectin were described previously.11,13

RT-PCR
Total cellular RNA of platelets from WT or APN-KO mice was obtained, and contaminated genomic DNA was removed using a QuantiTect Reverse-Transcription kit (QiAGEN). One microgram of total RNA was used as a template for RT-PCR as described previously.16 For the amplification of transcripts of mouse adiponectin receptors AdipoR1 and AdipoR2, the following primers were used: mouse AdipoR1 5'-ACGTTGAGAAGTCATCGGTAT-3' (sense) and 5'-CTCTGTGATGGCAGGAAAT-3' (antisense) and mouse AdipoR2 5'-TGGGACACATTTTCAGTCTCT-3' (sense) and 5'-TTCTATGATCCCCAAAGTTGTC-3' (antisense).19,20 For human platelet isolation, PRP obtained from 50 mL of whole blood was passed through a leukocyte removal filter as described previously.21 This procedure removed >99.9% of the contaminated leukocytes.21 For human AdipoR1 and AdipoR2, the following primers were used: human AdipoR1 5'-CTTCCTACTGCTCCCCACACGC-3' (sense) and 5'-GACAAACCTCAGCGTAGAT-3' (antisense) and human AdipoR2 5'-GGAGCGGAGCAAAAGCTCAG-3' (sense) and 5'-CACCCAGAGTCTGACTTCTC-3' (antisense). In addition, total cellular RNA obtained from a megakaryocytic cell line, CMK, and that from a human monocytic cell line, THP-1 (positive control)22 was examined in parallel. RT-PCR samples omitting reverse transcriptase were used as negative controls.

Statistical Analysis
Results were expressed as mean±SD. Differences between groups were examined for statistical significance using Student’s t test.

Results

Characteristics of Adiponectin-Deficient Mice and Assessment of Atherosclerotic Lesions
The basal profiles of APN-KO male mice have been previously described.10,12 To exclude the effects of diet on APN-KO mice, we used APN-KO male mice (8 to 12 weeks old) fed on a normal diet in this study. There were no differences in platelet counts, PT, APTT, and plasma fibrinogen concentrations (Table I, available online at http://atvb.ahajournals.org). Histological analyses revealed that neither Oil Red O staining of the inner surface of whole aorta nor elastin–van Gieson staining of transverse sections of carotid arteries showed any apparent atherosclerotic lesions in WT or APN-KO mice (data not shown).

Bleeding Time in APN-KO Mice
To examine the effects of adiponectin deficiency on thrombosis and hemostasis, we studied bleeding time in APN-KO mice. The bleeding time in APN-KO mice was slightly but significantly shorter (96.9±34.9 s; n=30; P<0.05) than that in WT mice (130.9±52.1 s; n=30).

Enhanced Thrombus Formation in APN-KO Mice and Adiponectin Adenovirus Ameliorates the Thrombogenic Tendency
We next examined the effect of adiponectin deficiency on thrombus formation using the He-Ne laser-induced carotid
artery thrombus model. Endothelial injury of the carotid artery was induced by the interaction of Evans blue dye with irradiation from the He-Ne laser. In WT mice, thrombus formation started 61.0 ± 100625.0 s after the initiation of He-Ne laser irradiation (n = 10). When the thrombi reached a certain size, they frequently ruptured and detached themselves from the wall because of increased shear stress. Thus, thrombus formation in this in vivo model showed a cyclic fluctuation, and complete occlusion was not observed (Figure 1). During a 10-minute observation period, the cycles of thrombus formation were 8.5 ± 10062.3 in WT mice. In APN-KO mice, there was no significant difference in the initiation time for thrombus formation (54.8 ± 10068.9 s; n = 10; P = 0.46). However, the cycles of thrombus formation during the 10-minute observation period were significantly fewer (5.4 ± 10062.0; n = 10; P < 0.01) in APN-KO mice. The thrombi in APN-KO mice grew larger and appeared to be stable and more resistant to the increased shear stress. Accordingly, the total thrombus volume was significantly larger in APN-KO mice (6.74 ± 10032.87 × 107 arbitrary units in WT mice and 13.36 ± 10034.25 × 107 arbitrary units in APN-KO mice; n = 10; P < 0.01).

To confirm that adiponectin deficiency is responsible for the enhanced thrombus formation in APN-KO mice, we injected Ad-βgal or Ad-APN into APN-KO mice. On the fifth day after adenoviral injection, we confirmed the elevated plasma adiponectin level in Ad-APN-infected APN-KO mice in an ELISA assay (48.7 ± 6.8 μg/mL; n = 4), as well as in an immunoblot assay. In the carotid artery thrombus model, the total thrombus volume in Ad-βgal-infected APN-KO was 12.94 ± 4.67 × 107 arbitrary units, which was compatible with that of APN-KO mice shown in Figure 1. In contrast, Ad-APN infection significantly decreased the total thrombus volume in APN-KO mice (6.23 ± 3.09 × 107 arbitrary units; n = 4; P < 0.05). These results indicate that adiponectin deficiency is responsible for the thrombogenic tendency in vivo.

Platelet-Thrombus Formation on Immobilized Collagen Under Flow Conditions

Because endothelial function may affect in vivo thrombus formation, we next performed in vitro mural thrombus formation on a type I collagen-coated surface under flow conditions. Figure 2 shows thrombus formation during a
10-minute perfusion of mouse whole blood anticoagulated with thrombin inhibitor at a low shear rate (250 s⁻¹). In whole blood obtained from WT mice, the thrombus fully covered the collagen-coated surface after 8 to 10 minutes of perfusion. In contrast, the thrombus grew more rapidly and fully covered the surface at 6 minutes in APN-KO mice. At 1 and 2 minutes of perfusion, there was no apparent difference in the initial platelet adhesion to the collagen surface between WT and APN-KO mice, whereas the platelet aggregate formation was significantly enhanced in APN-KO, even at 1 minute. We additionally examined the possibility that adiponectin might inhibit platelet adhesion onto collagen, because adiponectin binds to collagen types I, III, and V. However, mouse recombinant adiponectin (40 μg/mL) did not inhibit the adhesion of platelets onto collagen, indicating that the inhibitory effect of adiponectin is not mediated by the inhibition of platelet binding to collagen (data not shown). At a high shear rate (1000 s⁻¹), the thrombus grew rapidly and fully covered the surface within 3 to 4 minutes. Under such strong stimuli, we did not detect any difference in thrombus formation between WT and APN-KO mice, probably because of the full activation of platelets.

Adiponectin Inhibits the Enhanced Platelet Aggregation in APN-KO Mice

In platelet aggregation studies, PRP obtained from APN-KO mice showed significantly enhanced platelet aggregation in response to low doses of agonists (ADP 2.5 μmol/L, collagen 2.5 μg/mL, and protease-activated receptor 4–activating peptide [PAR4-TRAP] 75 μmol/L), as compared with WT mice (Figure 3). The maximal platelet aggregation was achieved at higher concentrations of agonists, and the enhanced platelet aggregation in APN-KO mice was not apparent at these high doses of agonists, probably because of the full activation of platelets.

To confirm the inhibitory effect of adiponectin on platelet aggregation in vitro, we mixed 1 volume of PRP obtained from APN-KO mice with 4 volumes of platelet-poor plasma (PPP) obtained from APN-KO mice injected with either Ad-beta-gal or Ad-APN to adjust platelet counts to 300×10⁹/μL. As shown in Figure 4A, the in vitro supplementation of PPP containing adiponectin attenuated the enhanced platelet aggregation. Similarly, in vitro administration of mouse recombinant adiponectin (40 μg/mL) to PRP from APN-KO mice attenuated the enhanced platelet aggregation (Figure 4B).

Expression of Adiponectin Receptors in Platelets and Effects of Adiponectin Deficiency on α₉β₃ Activation and P-Selectin Expression

To reveal the effect of adiponectin on platelets, we examined whether platelets possess transcripts for adiponectin receptors AdipoR1 and AdipoR2 by using RT-PCR. As shown in Figure 5A, platelets from APN-KO, as well as WT mice, contained mRNAs for AdipoR1 and AdipoR2. We also confirmed that the human megakaryocytic cell line CMK, as well as carefully isolated human platelets, possessed mRNAs for AdipoR1 and AdipoR2. We next examined the effects of adiponectin deficiency on α₉β₃ activation and α-granule secretion at various concentrations of agonists by flow cytometry. However, neither the platelet α₉β₃ activation induced by ADP nor P-selectin expression induced by PAR4-TRAP showed significant difference between WT and APN-KO mice (n=4; Figure 5B and 5C).

Adiponectin Adenovirus Attenuates Thrombus Formation in WT Mice

Because WT mice have large amounts of adiponectin in their plasma, we, therefore, examined whether adiponectin overexpression could additionally inhibit thrombus formation, as well as platelet function, in WT mice. After the administration of Ad-APN or Ad-beta-gal into WT mice, the plasma adiponectin levels in Ad-APN-infected mice reached ~4 times higher than those in Ad-beta-gal-infected WT mice (8.5±0.6 μg/mL for Ad-beta-gal and 37.0±14.8 μg/mL for Ad-APN; n=5). As shown in Figure 6A, platelet aggregation in PRP induced by collagen or PAR4-TRAP was significantly attenuated by the overexpression of adiponectin. Similarly, in vitro administration of human recombinant adiponectin (40 μg/mL) to human PRP attenuated the platelet aggregation response to 2.5 μg/mL collagen (Figure 6B). Moreover, in the He-Ne laser–induced carotid artery thrombus model, the overexpression of adiponectin significantly inhibited thrombus formation in WT mice (4.38±0.75×10⁷ arbitrary units for Ad-beta-gal and 2.75±0.61×10⁷ arbitrary units for Ad-APN; n=7; P<0.05; Figure 6C).
Discussion

In the present study, we have newly revealed an antithrombotic effect of adiponectin. APN-KO male mice (8 to 12 weeks old) fed on a normal diet showed no significant differences in platelet counts and coagulation parameters compared with WT mice. In the He-Ne laser–induced carotid artery thrombus model, APN-KO mice showed an accelerated thrombus formation, and adenovirus-mediated supplementation of adiponectin attenuated this enhanced thrombus formation. Platelet aggregometry and the real-time observation of in vitro thrombus formation on a type I collagen-coated surface under flow conditions showed the enhanced platelet function in APN-KO mice. Moreover, adenovirus-mediated supplementation of adiponectin attenuated this enhanced thrombosis formation. Platelet aggregometry and the real-time observation of in vitro thrombus formation on a type I collagen-coated surface under flow conditions showed the enhanced platelet function in APN-KO mice. Moreover, adenovirus-mediated overexpression of adiponectin attenuated in vivo thrombus formation, as well as the in vitro platelet aggregation response, even in WT mice. Thus, the present data strongly suggest that adiponectin possesses an antithrombotic potency.

We have demonstrated that low concentrations of adiponectin are associated with the prevalence of CAD in men, which is independent of well-known CAD risk factors. Pischon et al have recently shown that high concentrations of adiponectin are associated with a lower risk of myocardial infarction in men, which is also independent of inflammation and glycemic status and can be only partly explained by differences in blood lipids. These clinical studies suggest that the protective effect of adiponectin on the development of CAD may be primary rather than secondary through the protection of metabolic abnormalities, such as insulin resistance. Indeed, APN-KO mice fed on a normal diet did not show any abnormalities in plasma glucose, insulin, or lipid profiles. Although the atherosclerotic and thrombotic processes are distinct from each other, these processes appear to be interdependent, as shown by the term atherothrombosis. The interaction between the vulnerable atherosclerotic plaque, which is prone to disruption, and thrombus formation is the cornerstone of acute coronary syndrome (ACS). In this context, our present data strongly suggest that adiponectin deficiency (or hypoadiponectinemia) may directly contribute to the development of ACS by enhanced platelet thrombus formation.

Although APN-KO fed on a normal diet showed no significant differences in major metabolic parameters, they showed delayed clearance of FFA in plasma, elevated plasma TNF-α concentrations (∼40 pg/mL in APN-KO; ∼20 pg/mL in WT), and elevated CRP mRNA levels in white adipose tissue. In addition, recombinant adiponectin increased NO production in vascular endothelial cells.
Then become activated and aggregate to each other. The thrombus formed in APN-KO mice appeared to be stable and more resistant to the increased shear stress, without affecting the initiation time for thrombus formation in carotid artery injury experiments, as well as in flow chamber perfusion experiments. In addition, preincubation of collagen with recombinant adiponectin did not inhibit platelet adhesion on collagen under static conditions. Thus, it is unlikely that the inhibitory effect of adiponectin is mediated by the inhibition of platelet binding to collagen. These characteristics are quite distinct from C1q-TNF-related protein-1, which belongs to the same family as adiponectin and inhibits thrombus formation by interfering with platelet–collagen interaction. We confirmed that transcripts for AdipoR1 and AdipoR2 were present in mouse and human platelets and CMK cells. Although the platelet–platelet interaction appeared to be enhanced in APN-KO mice, we did not detect any difference in agonist-induced $\alpha_{IIb}\beta_3$ activation or P-selectin expression between APN-KO and WT mice by flow cytometry. Based on these results, it is possible that adiponectin may inhibit $\alpha_{IIb}\beta_3$-mediated intracellular postligand binding events. Alternatively, previous studies have shown that adiponectin is physically associated with many proteins, including $\alpha_{IIb}$-macroglobulin, thrombospondin-1 (TSP-1), and several growth factors. Interestingly, TSP-1, after secretion from platelet $\alpha$ granules, may participate in platelet aggregation by reinforcing interplatelet interactions through direct fibrinogen-TSP-fibrinogen and TSP-TSP crossbridges. In this context, it is also possible that it may interfere with interplatelet interactions in platelet aggregation. Additional studies to clarify the mechanism of adiponectin are currently under way.

In conclusion, our present study revealed that adiponectin acts as an endogenous antithrombotic factor. Although it is possible that the in vivo antithrombotic effect of adiponectin may be partly mediated by its action on vascular cells, our present data clearly indicate that adiponectin affects platelet function in the absence of vascular cells. In addition, the overexpression of adiponectin in WT mice attenuates in vivo thrombus formation, as well as the in vitro platelet aggregation response. Our data provide a new insight into the pathophysiology of ACS in nonobese, as well as obese, subjects, and adiponectin (and its derivatives) may be a new candidate for an antithrombotic drug.

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### Table I. Hematological features of APN-KO mice

<table>
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<th>APN-KO</th>
<th>n</th>
<th>p-value</th>
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<td>954</td>
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<td><strong>Fibrinogen</strong> (mg/dl)</td>
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<td>136.7</td>
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