is precedent and causative of hypertension and cardiovascular disease in metabolic syndrome.[33] In our recent analysis, hypoadiponectinemia was an independent risk factor of hypertension in human subjects, independent of obesity and insulin resistance.[20] In addition, we reported recently that subjects with I164T mutation of adiponectin gene, who exhibited remarkable hypoadiponectinemia, had higher prevalence of coronary artery disease and hypertension unrelated to obesity.[20,22] These findings suggest that hypoadiponectinemia contributes directly to the development of hypertension in humans. Next, we further investigated the role of adiponectin on blood pressure in the mouse model. Adiponectin KO mice did not display the phenotype of the metabolic syndrome under normal diet. On high-fat/high-sucrose diet, however, the KO mice developed severe insulin resistance.[10] In addition, the KO mice showed delayed clearance of free fatty acid in plasma and low levels of fatty-acid transport protein 1 mRNA in muscle, although no differences were observed in total cholesterol levels and triglyceride levels in plasma.[10,23] On high-fat/high-sucrose/high-salt diet, the KO mice developed hypertension and diabetes mellitus with impaired acetylcholine-induced vasorelaxation of aortic rings.[23] In the present study, we showed that obese KKAy mice had hypoadiponectinemia and that adiponectin supplementation ameliorated the hypertension in these mice. In addition, adiponectin KO mice developed hypertension without insulin resistance when maintained on a high-salt diet. These results suggest that hypoadiponectinemia, per se, is not sufficient for the development of hypertension but contributes to its development under insulin resistance and/or salt overload, although further studies are necessary to determine the blood pressure response to various doses of adiponectin.

In vascular endothelial cells, we have reported that adiponectin promoted the phosphorylation of AMP-activated protein kinase, protein kinase Akt/protein kinase B, and eNOS and that the adiponectin-AMP-activated protein kinase-Akt-eNOS signal was essential for the antiapoptotic and angiogenic effects.[24,25] It has been reported that some polymorphism of the human PGIS gene was an independent risk for systolic hypertension[34] and that PG I_2-deficient mice developed hypertension with the thickening of arterial walls.[35] Furthermore, an interaction between NO and PG pathways has been reported.[36] In this study, we demonstrated that adiponectin KO mice exhibited salt-induced hypertension accompanied by reduced mRNA levels of eNOS and PGIS in aorta and eNOS in kidney. In addition, adenoviral delivery of adiponectin improved the salt-induced hypertension and reversed the reduced mRNA levels of eNOS and PGIS in aorta of KO mice. In addition, L-NAME had no effect on SBP in adiponectin KO mice under a high-salt diet. On the other hand, there were no significant differences in plasma Na, Cl, K, angiotensin II, aldosterone, and leptin concentrations, in total urinary catecholamines, and in the mRNA levels of angiotensinogen and leptin in white adipose tissue, angiotensinogen in liver, and renin and epithelial sodium channel in kidney between salt-fed KO and WT mice, although it is possible that other mechanisms are involved in the development of hypertension. Thus, the present study suggests that the impaired adiponectin–eNOS–PGIS pathway in the systemic vasculature might be, at least in part, associated with the hypertension of salt-fed adiponectin KO mice, although further studies are necessary to elucidate the precise mechanism.

In conclusion, we demonstrated in the present study that adiponectin supplementation reduced blood pressure both in obese KKAy mice and salt-fed adiponectin KO mice without affecting the insulin-resistant state. Both KKAy mice and salt-fed adiponectin KO mice developed hypertension accompanied by reduced mRNA levels of eNOS in aorta and kidney and low NO metabolite levels in plasma. These results suggest that hypoadiponectinemia contributes to the development of obesity-related hypertension via a direct effect on vasculature, in addition to its effect on insulin resistance, and that adiponectin supplementation is a potentially useful therapeutic modality for hypertension, as well as insulin resistance in the metabolic syndrome.

Perspectives

Obesity is closely associated with hypertension. The mechanisms underlying hypertension in obesity, however, have not been fully clarified. Adiponectin has many defensive properties against obesity-related diseases, such as type 2 diabetes and coronary artery disease. In the present study, we demonstrated for the first time that adiponectin treatment lowers obesity-related hypertension. Therefore, adiponectin treatment is potentially useful for hypertension and may help in establishing a unified approach for the treatment of the metabolic syndrome.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (B) No. 14370327, Grants-in-Aid for Scientific Research on Priority Areas No. 13137206 and 15081208, Grant-in-Aid for Scientific Research (B) No. 15390287, Takeda Science Foundation, the Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists, Mitsubishi Pharma Research Foundation, Yamanouchi Foundation for Research on Metabolic Disorders, The Cell Science Research Foundation, and Japan Heart Foundation Grant for Research on Arteriosclerosis Update. We gratefully acknowledge the technical assistance of Sachiyo Tanaka and Kaori Yamamoto.

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Correction

In a *Hypertension* article by K Ohashi et al (Ohashi K, Kihra S, Ouchi N, Kumada M, Fujita K, Hiuge A, Hibuse T, Ryo M, Nishizawa H, Maeda N, Shibata R, Walsh K, Funahashi T, Shimomura I. Adiponectin replenishment ameliorates obesity-related hypertension. *Hypertension* 2006;47:1108–1116), there was a mistake in describing plasma adiponectin levels before adenovirus injection in Table 1. On day 11 post-injection of Ad-APN, plasma adiponectin concentrations were 56.8±5.0 μg/mL in KKAy/Ad-APN and 9.8±1.0 μg/mL in KKAy/Ad-β gal, as mentioned in the text. As a matter of course, the plasma adiponectin levels were significantly higher in KKAy/Ad-APN than in KKAy/Ad-β gal. Therefore, the correct Table 1 is shown here. The authors regret the error.

TABLE 1. Characterization of Ad-β gal- and Ad-APN–Treated KKAy Mice. Plasma Concentrations of Adiponectin, Fasting Plasma Glucose (FPG), Insulin RI (IRI), HOMA-IR, Total Cholesterol (T-chol), Triglyceride (TG), Angiotensin II, Aldosterone, and Leptin Concentrations in Ad-β gal- and Ad-APN-Treated KKAy Mice

Variables	Ad-β gal (n=9)	Ad-APN (n=9)	*P*
Adiponectin, μg/mL	9.8±1.0	56.8±5.0	<0.01
FPG, mmol/L	10.35±0.82	11.16±0.82	NS
IRI, μU/mL	392.8±88.9	352.3±38.8	NS
HOMA-IR	183.0±20.1	197.3±17.1	NS
T-chol, mmol/L	3.53±0.28	3.36±0.17	NS
Triglyceride, mmol/L	1.55±0.16	1.67±0.15	NS
Angiotensin II, pg/mL	172.7±83.5	174.0±75.2	NS
Aldosterone, pg/mL	488.8±105.0	412.7±147.1	NS
Leptin, ng/mL	34.88±2.81	31.08±2.76	NS

NS, not significant. Data are mean±SEM.

(*Hypertension*. 2007;49:e14)

Hypertension is available at http://www.hypertensionaha.org DOI: 10.1161/01.HYP.0000255633.14303.3a

0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(4):1539–1549

doi: 10.1210/en.2006-0838

Overexpression of Adiponectin Targeted to Adipose Tissue in Transgenic Mice: Impaired Adipocyte Differentiation

Isabelle B. Bauche,* Samira Ait El Mkadem,* Anne-Marie Pottier, Maximin Senou, Marie-Christine Many, René Rezsohazy, Luc Penicaud, Norikazu Maeda, Tohru Funahashi, and Sonia M. Brichard

Endocrinology and Metabolism Unit (I.B.B., S.A.E.M., A.-M.P., S.M.B.), Experimental Morphology Unit (M.S., M.-C.M.), University of Louvain, Faculty of Medicine, 1200 Brussels, Belgium; Unit of Veterinary Sciences (R.R.), Institut des Sciences de la Vie, University of Louvain, 1348 Louvain-la-Neuve, Belgium; Centre National de la Recherche Scientifique (CNRS) Unité Mixte de Recherche 5018 (L.P.), CNRS and Paul Sabatier University, 31062 Toulouse, France; and Department of Internal Medicine and Molecular Science (N.M., T.F.), Graduate School of Medicine, Osaka University, Osaka 560-0043, Japan

Adiponectin (ApN) is an adipokine whose expression and plasma levels are inversely related to obesity and insulin-resistant states. Chronic repercussions of ApN treatment or overexpression on adiposity and body weight are still controversial. Here, we generated a transgenic (Tg) mouse model allowing persistent and moderate overexpression of native full-length ApN targeted to white adipose tissue. Adipose mass and adipocyte size of Tg mice were reduced despite preserved calorie intake. This reduction resulted from increased energy expenditure and up-regulation of uncoupling proteins, and from abrogation of the adipocyte differentiation program, as shown by the loss of a key lipogenic enzyme and of adipocyte markers. Adipose mass remodeling favors enhanced insulin sensitivity and improved lipid profile of Tg mice. Alteration of the adipocyte phenotype was likely to result from increased expression of the preadipocyte factor-1 and from down-regulation of the transcription factor, CCAAT/enhancer binding protein-α, which orchestrates adipocyte differentiation. We further found that recombinant ApN directly stimulated preadipocyte factor-1 mRNA and attenuated CCAAT/enhancer binding protein-α expression in cultured 3T3-F442A cells. Conversely, opposite changes in the expression of these genes were observed in white fat of ApN-deficient mice. Thus, besides enhanced energy expenditure, our work shows that impairment of adipocyte differentiation contributes to the anti-adiposity effect of ApN. (*Endocrinology* 148: 1539–1549, 2007)

ADIPONECTIN (ApN) IS AN adipokine, specifically secreted by adipocytes, that circulates at relatively high concentrations in the bloodstream. It plays a fundamental role in energy homeostasis and inflammation (1). This 30-kDa protein is composed of an N-terminal collagenous domain and a C-terminal globular domain. The latter fragment [globular ApN (gApN)] generated by proteolysis may exert some activity (2). Two types of ApN receptor mediate most effects of the hormone via stimulation of AMP kinase, peroxisome proliferator-activated receptor (PPAR) α, and p38 MAPK. ApN receptor 1 (AdipoR1), which is a high-affinity receptor for gApN, is most abundantly expressed in skeletal muscle, whereas ApN receptor 2 (AdipoR2), which serves as a moderate-affinity receptor for both forms of ApN, is predominant in liver (3). Both receptor types are present in adipocytes, which suggests that ApN may act on these cells in an autocrine or paracrine manner (4, 5).

Unlike most adipocytokines, circulating ApN is decreased in human obesity and related disorders (type 2 diabetes or cardiovascular disease, which are components of the metabolic syndrome) (6–9). ApN supplementation could carry significant therapeutic potential in these adverse metabolic events, as shown in mice. ApN treatment plays a protective role against atherosclerosis in apolipoprotein E-deficient mice (10). The adipocytokine is also a potent enhancer of insulin action in mouse models of obesity, lipoatrophy, or diabetes (11, 12). Its administration induces insulin-dependent suppression of hepatic gluconeogenesis, thereby lowering plasma glucose concentrations (11). It also increases glucose uptake by muscles (13, 14). ApN alters lipid metabolism as well, by increasing fatty-acid oxidation in several tissues including liver and muscle, thereby accelerating the clearance of plasma free fatty acids (2, 13–15). Concomitantly, ApN stimulates the expression of uncoupling proteins (UCPs) in various organs and enhances thermogenesis (12, 16, 17).

Our understanding of ApN action on adipose tissue is still

First Published Online January 4, 2007

* I.B.B. and S.A.E.M. contributed equally to this work.

Abbreviations: ACO, Acyl-coenzyme A oxidase; aP2, adipocyte P2; ApN, adiponectin; AdipoR1, ApN receptor 1; AdipoR2, ApN receptor 2; BAT, brown adipose tissue; C/EBP-α, CCAAT/enhancer binding protein-α; cyclo, cyclophilin; Ct, cycle threshold; FAS, fatty acid synthase; gApN, globular ApN; GLUT4, insulin-sensitive glucose transporter; HES, hematoxylin-eosin-safran; HMM, high molecular mass; LMM, low molecular mass; NEFA, nonesterified free fatty acids, OGTT, oral glucose tolerance test; Pref-1, preadipocyte factor-1; PPAR, peroxisome proliferator-activated receptor; RTQ-PCR, real-time quantitative PCR; SREBP-1, sterol regulatory element-binding protein-1; Tg, transgenic or transgene; UCP1, 2, 3, uncoupling protein 1, 2, 3; WAT, white adipose tissue; WT, wild type.

Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

poor. *In vitro*, ApN acutely stimulated glucose uptake by isolated rat adipocytes (18), and suppressed IL-6 and TNFα expression in pig adipocytes (19). Recently, marked overexpression of ApN in stably transduced 3T3-L1 cells has been shown to result in adipocyte differentiation and lipid accumulation (20). The *in vivo* effects of ApN treatment on mouse adiposity and body weight remain controversial: some studies reported a decrease in fat mass (2, 16, 17), whereas others reported no change or even an increase (12, 21). Moreover, ApN was usually delivered by infection with a recombinant adenovirus or injection of purified recombinant protein, and experiments were conducted on a short or middle term basis (from a few days up to 3 wk). A good way to investigate the repercussions of chronic and early ApN supplement on adipose tissue is to generate appropriate Tg mouse models. It has been extremely difficult to elevate native ApN in adipose tissue of Tg mice because of a negative feedback exerted on endogenous production (21, 22). Two Tg models overexpressing ApN have been generated so far. In the first model, it was necessary to develop a particular Tg strategy using a deletion mutant of ApN to circumvent this problem. This mutant of full-length ApN cDNA was then placed under control of the adipocyte promoter (aP2) (21). In the second model, gApN cDNA was placed under control of a liver promoter (23). In the former case, an unexpected obese phenotype was observed, but the physiological relevance of this model bearing a deletion in the collagenous domain of ApN may not be straightforward (21). In the latter case, effects on body weight were variable (unchanged or decreased), but circulating gApN was very high and influenced other organs, so that repercussions on adipose tissue could be masked or indirectly mediated (23).

We generated a heterozygous Tg mouse line overexpressing native ApN specifically in white adipose tissue (WAT) to study the chronic effects of a local, additional but homotopic expression of ApN *in vivo*. This line carried approximately 100 copies of a transgene, in which native full-length ApN was placed under control of aP2. Previously characterized mouse lines that carried only six copies of this transgene failed to overexpress ApN (22). In the present line carrying a larger number of copies, ApN overexpression masks the down-regulation of the endogenous gene. Thanks to this high-expression line, we more particularly examined the chronic repercussions of ApN on body weight and fat accumulation.

Materials and Methods

Generation of Tg mice

A fusion gene was designed, comprising the 5.4-kb aP2 promoter fragment (kindly provided by B. M. Spiegelman, Dana-Farber Cancer Institute, Boston, MA) (24), the 1276-bp mouse full-length adiponectin (ApN) cDNA [gift of P. E. Scherer, Department of Cell Biology and Diabetes Research and Training Center, Albert Einstein College of Medicine, Bronx, New York (25)] and the simian virus 40 polyadenylation signal (Fig. 1A). The integrity of this 8-kb construct was confirmed by mapping with restriction endonucleases and DNA sequencing of crucial regions (aP2-ApN junction and ApN cDNA). The transgene was purified and microinjected into the pronucleus of fertilized FVB mice eggs (Eurogentec, Liège, Belgium).

Tg mice were screened by PCR amplification of tail genomic DNA with aP2 promoter-specific (sense, 5′-AGTCAAAACAGGAACCTT-TAAAATACTC-3′) and ApN cDNA-specific (antisense, 5′-AGAAAGCCAGTAAATGTAGAG-3′) oligonucleotides, which give rise to a 1065-bp product. Identification of Tg founder F_0 mice and quantification of transgene copies were further achieved by Southern blot analysis of *Hin*dIII-digested genomic DNA hybridized with a PCR probe amplified from the exon 3 of ApN (Fig. 1A). For this experiment, the filters were exposed to autoradiographic films after hybridization with the radiolabeled probe. ODs of the transgene band on the blots were quantified by scanning densitometry (Image Master TotalLab; Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK) and expressed relative to the OD of the band corresponding to the endogenous locus.

Eight F_0 mice had integrated the transgene, but only three of these were fertile. Two carried a low copy number of the transgene, which did not allow overexpression of ApN (22). The third one carried approximately 100 copies. The reported analysis focused on the progeny of this last line. Tg mice were maintained on a pure (C57/Bl6J) background and used as heterozygotes.

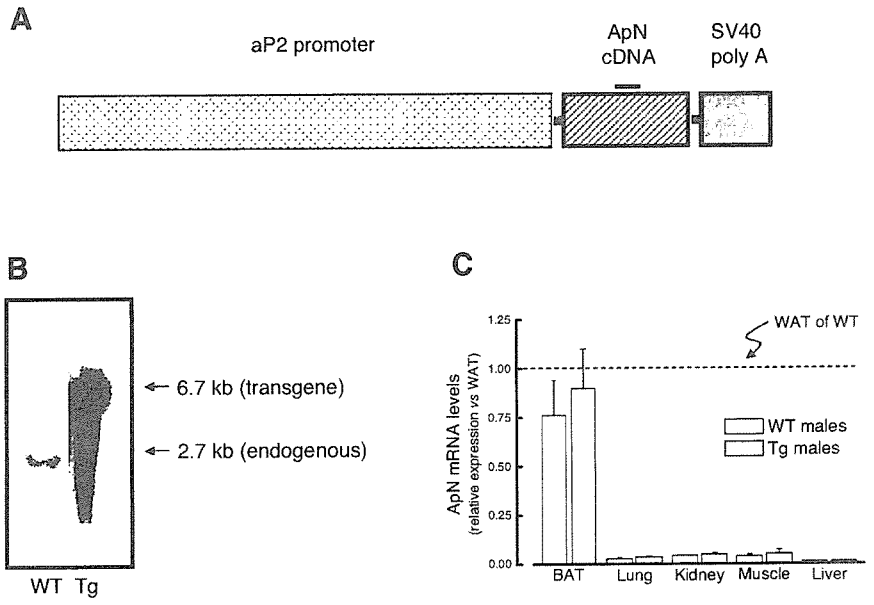

FIG. 1. Generation of mice carrying 100 copies of the ApN transgene under control of the adipocyte-specific aP2 promoter. A, Schematic representation of the aP2 promoter/mouse ApN cDNA fusion gene. The *bar* represents the DNA probe (fragment of exon 3) used for Southern blotting. B, Southern blot analysis of *Hin*dIII-digested mouse genomic DNA from WT and Tg mice hybridized with the cDNA probe shown in panel A. The 6.7-kb band corresponds to the transgene and the 2.7-kb band to the endogenous gene. C, ApN gene expression in several non-WATs of WT and Tg male mice. ApN mRNA levels were measured in BAT and in nonfat tissues. Data from 40-ng total RNA equivalents were quantified by RTQ-PCR, normalized to the levels of cyclophilin, and then presented as relative expression compared with the values obtained in WAT (inguinal depot) of WT mice (*dotted line*). Results are the mean ± SEM for six to eight mice per group.

Animal care, sampling, and tests

Tg mice and their wild-type (WT) littermates were housed in groups of two to five in filter-top cages with a fixed 12-h light, 12-h dark cycle. They received from weaning a high-sucrose diet that promotes insulin resistance. This diet was composed of (% of total gross energy): 68 carbohydrate (cornstarch/sucrose, 1:3.5), 11 fat (milk fat/soybean oil, 3:1), 21 protein (casein) (TD00220; Harlan, Horst, The Netherlands). In an additional experiment, some ApN-deficient mice, which are characterized by a lack of ApN mRNA in fat and ApN protein in plasma, were obtained from Maeda *et al.* (26); these animals and their WT controls were housed as described above and received a common laboratory chow (RO3-10; Safe, Villemoisson-sur-Orge, France).

Food intake and body weight were measured daily. On several occasions, tail vein blood collected from fed animals (between 0800 and 0900 h). Some mice underwent an oral glucose tolerance test (OGTT) or an insulin tolerance test after an overnight fast. The tests started at 0800 h. For the OGTT, glucose (30% in water) was introduced into the stomach through a fine gastric catheter at a dose of 2 g/kg body weight. For the insulin tolerance test, Actrapid^{R} (Novo Nordisk A/S, Bagsvaerd, Denmark) was injected ip at a dose of 0.75 U/kg body weight. Blood samples were obtained at 0, 30, 60, 120, and 180 min. At the age of 5 months, mice were killed by decapitation (between 0800 and 1000 h). Pairs of white fat pads from several depots [sc (inguinal), gonadal (epididymal and parametrial), and prerectal-ip (also refereed to elsewhere as "retrovesical") (22, 27)] as well as interscapular brown adipose tissue (BAT) were immediately removed, weighed, frozen in liquid nitrogen, and stored at −80 C.

The University Animal Care Committee has approved all procedures.

Culture of 3T3-F442A adipocytes

Mouse 3T3-F442A preadipocytes were grown at 37 C in 5% CO_2 in basal medium [*i.e.* DMEM with 1 g/liter glucose containing 10% fetal calf serum, 8 mg/liter biotin and 1% (vol/vol) of a commercial mixture containing penicillin and streptomycin (Invitrogen Life Technologies, Merelbeke, Belgium)], as described (27). Two days after confluence (d 0), adipocyte differentiation was initiated by addition of 17 nM insulin, 2 nM T_3, 100 nM dexamethasone, and 100 μM isobutylmethylxanthine for 48 h. Then, cells were refed by basal medium containing 17 nM insulin and 2 nM T_3 (27). The medium was changed every 24 h. At d 3, more than 90% of the cells had changed their morphology and more than 70% had accumulated fat droplets as evidenced by Oil Red O staining. These cells were cultured with or without mouse recombinant ApN [full-length form (22)] added to the medium at a final concentration of 1 μg/ml from d 0–3.

Real-time quantitative PCR (RTQ-PCR)

RNA was isolated from mouse tissues and cultured cells by TriPure Isolation Reagent (Roche Diagnostics, Vilvoorde, Belgium) (28). mRNAs were quantified by RTQ-PCR (27). Two micrograms of total RNA were reverse transcripted using oligo (deoxythymidine) primers and Superscript II Rnase H^{-} Reverse Transcriptase (Invitrogen Life Technologies). RTQ-PCR primers were designed (Primer Express Software, Applied Biosystems) for ApN (these primers were ApN cDNA-specific and recognized both endogenous ApN and transgene-derived ApN), AdipoR1, AdipoR2, aP2, fatty acid synthase (FAS), UCP1, 2, 3, acyl-coenzyme A oxidase (ACO) PPARα and γ, sterol regulatory element-binding protein (SREBP)-1, GLUT4, TNFα, leptin, preadipocyte factor (Pref)-1, CCAAT/enhancer binding protein-α (C/EBP-α) and cyclophilin (cyclo) (Table 1). Forty nanograms of total RNA equivalents were amplified with iQ Syber Green Supermix (Bio-Rad Laboratories, Nazareth, Belgium) containing 300 nM of each specific primer, using iCycler iQ Real Time PCR Detection System (Bio-Rad). PCR efficiency was approximately 1. The threshold cycles (Ct) were measured in separate tubes and in duplicate. The identity and purity of the amplified product were checked by electrophoresis on agarose mini-gels and analysis of the melting curve carried out at the end of amplification. To ensure the quality of the measurements, each plate included a negative control for each gene. The ΔCt values were calculated in every sample for each gene of interest as followed: $Ct_{\text{gene of interest}} - Ct_{\text{reporter gene}}$ with cyclophilin whose mRNA levels did not differ between control and test groups, as the reporter gene. Relative changes in the expression level of one specific gene ($\Delta\Delta$Ct) were calculated as ΔCt of the test group minus ΔCt of the control group, and then presented as $2^{-\Delta\Delta Ct}$.

Indirect calorimetry

Twenty-four-hour energy expenditure was measured by indirect calorimetry (Oxylet, Panlab-Bioseb, Chaville, France). The mice were housed in individual metabolic chambers, in which they were accustomed for at least 12 h before starting the measurements. They had free access to food and water. The temperature in the metabolic cage (23 ± 1 C) was stable and controlled throughout the experiment. The following metabolic parameters were measured: oxygen consumption (VO_2), carbon dioxide production (VCO_2), energy expenditure (calculated according to the following formula = $(3.815 + 1.232 \times VO_2/VCO_2) \times VO_2$), and locomotor activity. Oxygen consumption and carbon dioxide production were recorded at 5-min intervals using a computer-assisted data acquisition program (Chart 5.2; AD Instruments, Sydney, Australia) over a 24-h period and data were averaged for each mouse. Physical activities of the mice were monitored by an infrared photocell beam-interruption method (Panlab-Bioseb, Chaville France). Computer-assisted processing of respiratory exchanges and spontaneous activity signals made it possible to compute that part of the total metabolic rate devoted to fuelling the energy cost of activity. Thus, by continuously extracting the energy expended with activity, it was possible to compute the resting metabolism of these free-moving mice.

Immunohistochemistry

Adipose tissue was fixed in 10% formaldehyde for 24 h and embedded in paraffin. The 5 μm-thick sections were stained with hematoxylin-eosin-safran (HES). For immunohistochemistry, sections were processed

TABLE 1. Murine gene sequences (5′–3′) used as forward and reverse primers for RTQ-PCR

Gene	Sense primer	Antisense primer
ApN	GCAGAGATGGCACTCCTGGA	CCCTTCAGCTCCTGTCATTCC
AdipoR1	AACGGGCCATCCATTTTTG	TTAGCCGGGCTACATCAAGG
AdipoR2	AGTGTTTTCAGCACGCCCTC	GCTGAGCTCCACGGATTCTT
aP2	TGGGAGTGGGCTTTG	TGTCGTCTGCGGTGAT
FAS	GTGAAGAAGTGTCTGGACTGTGTCAT	TTTTCGCTCACGTGCAGTTTA
UCP2	AATCTCGGGAGGCACCTTTC	GAGAATGGGACTGGGCAGAG
ACO	TCTTCTTGAGACAGGGCCCA	GTTCCGACTAGCCAGGCATG
PPARα	CCCTGAACATCGAGTGTCGA	AATAGTTCGCCGAAAGAAGCC
PPARγ	TTGACCCAGAGCATGGTGC	GAAGTTGGTGGGCCAGAATG
SREBP-1	CCGGCTATTCCGTGAACATC	CAAGGGCATCTGAGAACTCCC
Glut4	GCCCCACAGAAGGTGATTGA	AGCGTAGTGAGGGTGCCTTG
TNFα	GCCACCACGCTCTTCTGTCT	GTCTGGGCCATGGAACTGAT
Leptin	TTCAAGCAGTGCCTATCCAGAA	GGATACCGACTGCGTGTGTG
C/EBPα	AACAGCAACGAGTACCGGGT	CACCTTCTGTTGCGTCTCCAC
Pref-1	GCCGGGGGCTAGCCCCGTGCAGG	AGGTGGTCATGTCAATCTTCTCGG
Cyclophilin	AACCCCACCGTGTTCTTC	TGCCTTCTTTCACCTTCCC

as previously described (29) using rabbit polyclonal antibodies directed against ApN (Chemicon, Biognast, Hesle, Belgium) and caspase-6 (Santa Cruz Biotechnology, Tebu-Bio, Baeckout, Belgium). Binding of antibodies was detected by applying for 30 min at room temperature a second (goat antirabbit) antibody conjugated to peroxidase-labeled polymer (En vision +; Dako, Copenhagen, Denmark). Staining was specific because there was no labeling when adipose tissue was incubated with preimmune serum used as control (not shown).

Morphometry

The mean relative proportion of adipocytes was estimated by a point-counting technique (30) on paraffin-embedded, HES counterstained sections of inguinal tissue from Tg mice and their littermates. The number of adipocytes per microscopical field (density) was determined, at magnification ×500, on 20 fields for each mouse. Adipocytes (500–800) were counted for each section. The mean diameter of the adipocytes (in micrometers) was calculated as $\sqrt{}$(number of fields/ number of adipocytes) × D, with D being the diameter of the microscopical field (220 μm for the objective 50).

Quantification of total plasma ApN and determination of oligomers

Total plasma ApN concentrations were measured by a commercially available RIA kit (RIA mouse ApN kit; Linco Research, St. Charles, MO) as described (31). Samples (0.5 μl) were run in duplicate.

For separation of plasma ApN complexes, samples (0.33 μl) were solubilized in Laemmli buffer and subjected to SDS-PAGE under nonreducing and non-heat-denaturating conditions. Alternatively, samples were heated at 100 C for 5 min in the presence of 5% 2-mercaptoethanol for complete reduction and heat denaturation. Immunoblotting was performed using a rabbit polyclonal antibody directed against murine ApN (BioVendor Laboratory Medicine, Heidelberg, Germany; final concentration 1 μg/ml). After reaction with a secondary antibody (antirabbit IgG-hoseradish peroxidase), the blots were treated with enhanced chemiluminescence (SuperSignal West Dura Extended Duration Substrate, Pierce Science, Antwerpen Belgium) and analyzed using the Kodak Image Station system (Analis, Suarlee, Belgium).

Other analytical procedures

Blood glucose was measured using a glucometer (Medisense Precision Xtra Plus, Abott-Medisense, Louvain-la-Neuve, Belgium) and plasma insulin by RIA (kit from Linco Research). The insulin resistance indices were calculated as the insulin and glucose products derived from values obtained at the end of the OGTT (t180) (32). Plasma lipids [total cholesterol, nonesterified free fatty acids (NEFA), and triglycerides] were measured as reported (27).

Statistical analysis

Results are the means ± SEM for the indicated numbers of individual mice (*in vivo* study) or separate experiments (*in vitro* study). Comparisons between the two groups of mice or two different culture conditions were made using two-tailed unpaired Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

Results

Generation of mice carrying the aP2-ApN-Tg

We generated a heterozygous Tg mouse line to investigate the chronic repercussions of ApN overexpression on adiposity. Native full-length ApN was placed under the control of aP2, so that the hormone expression should be targeted mainly to adipose tissue (Fig. 1A). We obtained one line, which carried 100 copies of the transgene as assessed by Southern blotting (Fig. 1B). The 2.7-kb band corresponded to the endogenous gene and the 6.7-kb band to the aP2-ApN transgene. In this line, ApN gene overexpression was restricted to WAT. Indeed, ApN mRNA levels did not significantly differ in BAT of WT and Tg mice and were similarly low ($\leq$ 0.05) in the other tissues of the two groups, when compared with the amount found in WAT of WT mice (Fig. 1C).

Overexpression of ApN in Tg mice

As expected, ApN mRNA levels increased by 50–150% in almost every white fat depot of Tg males and females when compared with WT littermates (Fig. 2A). Accordingly, immunohistochemistry clearly demonstrated that ApN protein labeling was augmented in white fat of Tg mice. Immunostaining of adipose tissue sections showed that ApN was localized in the cytoplasmic edging with the periphery of the adipocytes and that labeling was much more intense in Tg animals (Fig. 2B; inguinal fat of males as an example).

In agreement with adipose tissue content, total plasma ApN levels rose 2- to 10-fold in Tg mice. The largest increase was observed in Tg females (Fig. 2C). ApN multimers were analyzed by SDS-PAGE under nonreducing and non-heat-denaturating conditions, which allows adequate separation of high molecular mass (HMM) and low molecular mass (LMM) complexes (33). As described (33), ApN circulates as a HMM form greater than 300 kDa, corresponding to a multimeric assembly of 12–16 molecules, and a LMM hexamer of approximately 150 kDa (Fig. 2D). Under reducing and heat-denaturating conditions, the multimeric circulating forms were converted to a 30-kDa monomer (Fig. 2D). Although the absolute levels of both HMM and LMM multimers increased in Tg mice, there was no major change in the multimer distribution between Tg and respective WT animals (Fig. 2D).

Glucose homeostasis and circulating lipid profile

Because ApN exerts antidiabetic properties, we examined glucose homeostasis. In the basal state, fed blood glucose levels were reduced by approximately 5–15% in Tg mice of both genders, and fed plasma insulin were reduced as well (Fig. 3, A and B). During the OGTT, blood glucose and plasma insulin levels were also lower in Tg than in WT mice at some time points (Fig. 3, C and D). The insulin resistance indices derived from values obtained at the end of the OGTT were decreased by approximately 35% in Tg mice [Fig. 3C, *inset* for females; similar data obtained for males (data not shown)]. An insulin tolerance test confirmed the enhanced insulin sensitivity (data not shown).

The plasma lipid profile was also modified in Tg mice (Table 2). In agreement with other reports (2, 21, 23), plasma levels of triglycerides, cholesterol, and NEFA were significantly reduced in Tg animals.

Body weight, adiposity, and energy intake and expenditure in Tg mice

As shown in Fig. 4A, body weight decreased significantly in Tg mice of both genders from 3 months of age onwards. At 5 months, white fat was sampled in several depots. Weight of every fat pad was reduced in Tg mice, with the largest reduction being observed in the inguinal depot (~−55%) (Fig. 4B).

Surprisingly, daily food consumption was unaltered in Tg

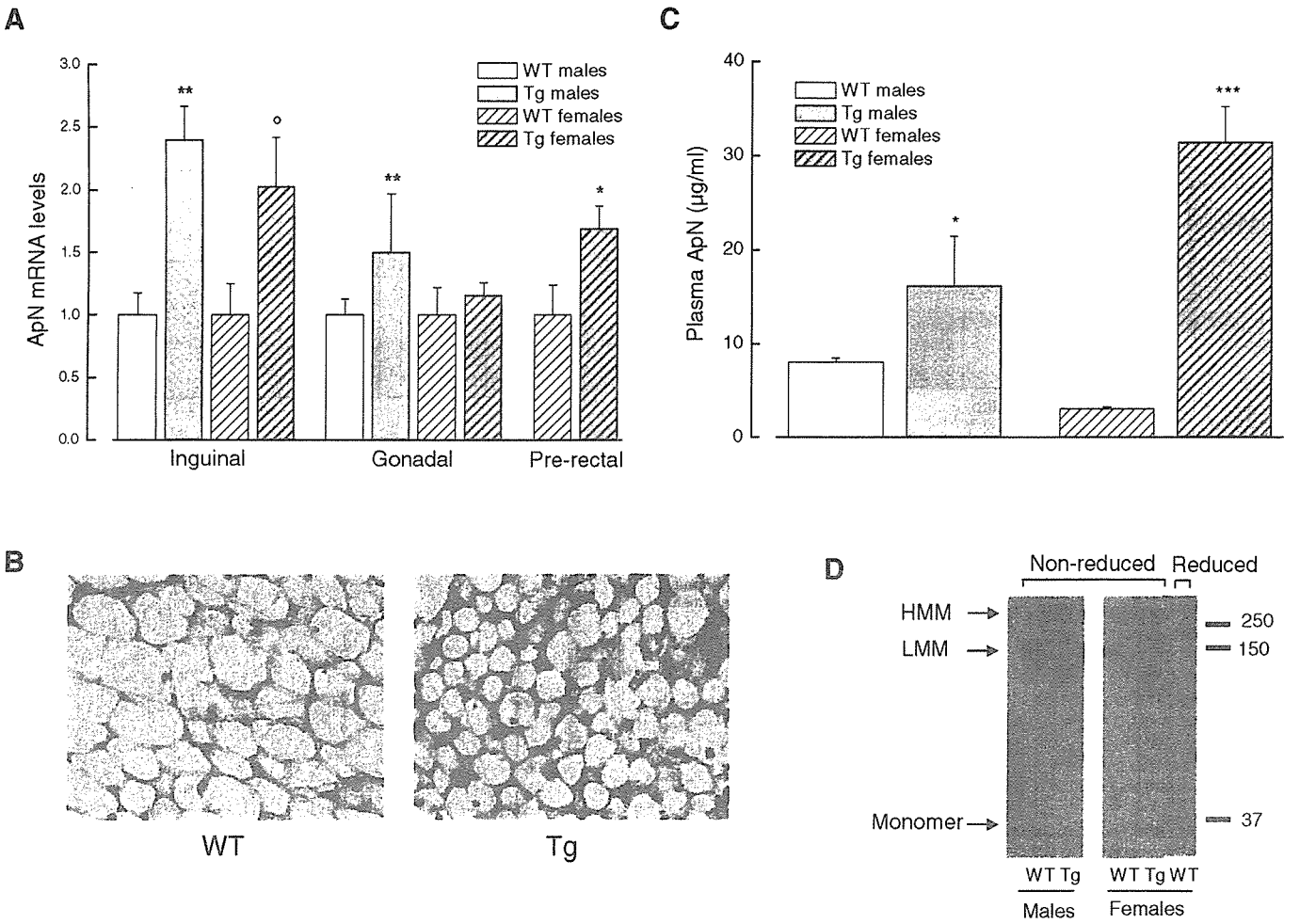

FIG. 2. Gene expression and immunodetection of ApN in adipose tissue, and levels of ApN in plasma of Tg mice. Five-month-old Tg and WT mice were used. A, ApN mRNA levels were measured in several fat depots by RTQ-PCR as described in Fig. 1 and are presented as relative expression compared with respective WT values. Due to limited amounts of prerectal fat in males (see Fig. 4B), ApN mRNAs of this depot were measured in females only. B, Representative sections of ApN immunodetection in inguinal adipose tissue are shown (at magnification, ×500). C, Total plasma ApN concentrations were measured by RIA and expressed as micrograms per milliliter. D, Representative Western blots showing HMM multimers migrating above 300 kDa and LMM hexamers migrating at approximately 150 kDa under nonreducing and non-heat-denaturating SDS-PAGE conditions. Separate gels were performed for males and females. Values are the mean ± SEM for eight mice per group (a and c). °, $P = 0.09$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* WT mice.

mice or even slightly elevated in females (by 10%) (Table 3). As a result, feed efficiency was decreased in Tg animals of both genders. A potential explanation for this finding could be an increase in the metabolic rate. To test this hypothesis, we measured 24-h energy expenditure by indirect calorimetry in 5-month-old mice. As shown in Table 3, energy expenditure was significantly higher in Tg than in WT mice. Locomotor activity was not influenced by the presence of the transgene (not shown).

Characteristics of fat in Tg mice: morphometry and expression of key molecules involved in adipose tissue metabolism and development

Histological examination of inguinal adipose tissue sections after HES staining showed a higher density of smaller adipocytes in Tg mice (Fig. 4C). Morphometric analysis confirmed that adipocyte size (diameter in micrometers) was halved in Tg animals compared with WT littermates (39 ± 0.6 *vs.* 61.3 ± 1.4 for males; 35.6 ± 0.4 *vs.* 60.2 ± 1.2 for females; n = 5 per group; $P < 0.05$ or less). In addition, adipocyte number per microscopical field (objective 50) was ~2.5-fold higher in Tg than in WT mice (33.5 ± 0.8 *vs.* 14.5 ± 0.7 for males; 39.6 ± 0.9 *vs.*15.2 ± 0.5 for females; $P < 0.0001$ for each comparison). However, because fat mass was reduced in Tg mice (Fig. 4), the estimated number of total adipocytes per animal was unlikely to be markedly altered in this model.

Gene expression of molecules involved in adipose tissue metabolism and development was next quantified in fat depots of 5-month-old Tg males. Because of increased energy expenditure, we measured the expression of UCPs, molecules involved in energy dissipation. Abundance of UCP 2 mRNA, the isoform expressed at a high level in white fat, doubled in the inguinal depot of Tg mice (Fig. 5, *left panel*). UCP 1 mRNAs did not change in this depot, but tended to

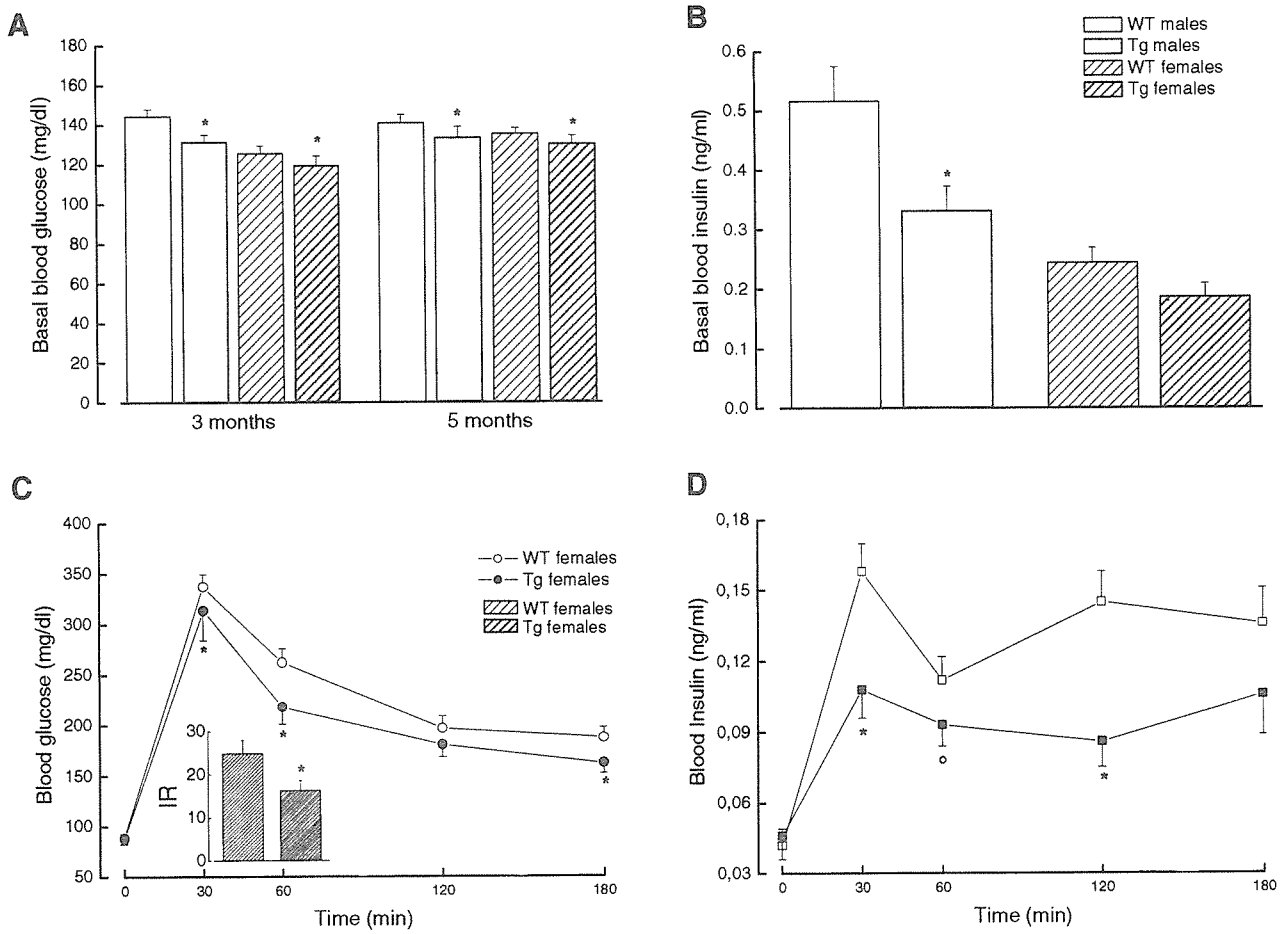

FIG. 3. Glucose homeostasis of Tg mice. A and B, Basal blood glucose and plasma insulin were sampled in the fed state in 3- and 5-month-old animals (only 3 months old for insulin). C and D, Blood glucose (G) and plasma insulin levels during an OGTT performed in 3-month-old mice after an overnight fast. *Inset*, IR (insulin resistance) indices [I (ng/ml) × G (mg/dl)] derived from the OGTT. Values are the mean ± SEM for eight to nine mice per group. °, $P = 0.08$; *, $P < 0.05$ *vs.* WT mice.

rise in epididymal fat (by 80%; $P = 0.08$) and BAT as well (by 40%; $P = 0.07$). UCP 3 expression, which predominates in skeletal muscle, was not significantly increased in tibialis anterior muscle (data not shown). Concomitantly with the increase of UCP2 expression, mRNA levels of FAS, a key enzyme of lipogenesis and of TNFα, an adipokine implicated in insulin resistance were decreased by 50 and 60% in inguinal fat of Tg mice. Leptin mRNAs were also reduced (Fig. 5, *left panel*). The expression of other messengers involved in glucose or lipid metabolism (Glut 4, aP2, and ACO) did not change (data not shown).

We measured the expression of ApN receptors in inguinal fat of Tg mice as well. AdipoR1 mRNAs did not vary, whereas AdipoR2 mRNAs increased 3-fold when compared with WT mice (Fig. 5, *left panel*).

Because the adipose tissue of Tg mice was characterized by younger and smaller adipocytes, we searched for changes in transcription/growth factors orchestrating adipose tissue development (Fig. 5, *right panel*). The expression of Pref-1, an epidermal growth factor implicated in the maintenance of the preadipose state (34), was increased in inguinal fat of Tg mice, whereas mRNA levels of C/EBPα, a factor inducing adipocyte gene expression and differentiation (35), tended to be decreased (but this trend did reach statistical significance; $P = 0.06$). The presence of the transgene did not affect the expression of other transcription factors also implicated in adipose tissue development, such as SREBP, PPARα, and PPARγ (36).

Eventually, reduced fatness in Tg mice did not result from

TABLE 2. Plasma lipid levels in Tg mice

	Males		Females	
	WT	Tg	WT	Tg
Triglycerides (mg/dl)	128.7 ± 14.9	95.5 ± 6.4[a]	113 ± 10.1	86.7 ± 10.8[a]
Cholesterol (mg/dl)	189.9 ± 8.4	165.5 ± 8.6[a]	162.7 ± 9	129.2 ± 17.5[a]
NEFA (mmol/liter)	0.119 ± 0.02	0.089 ± 0.02[a]	0.166 ± 0.02	0.126 ± 0.03

Measurements were made in 3-month-old WT and Tg mice. Results are the mean ± SEM for eight mice per group.
[a] $P < 0.05$ *vs.* WT mice.

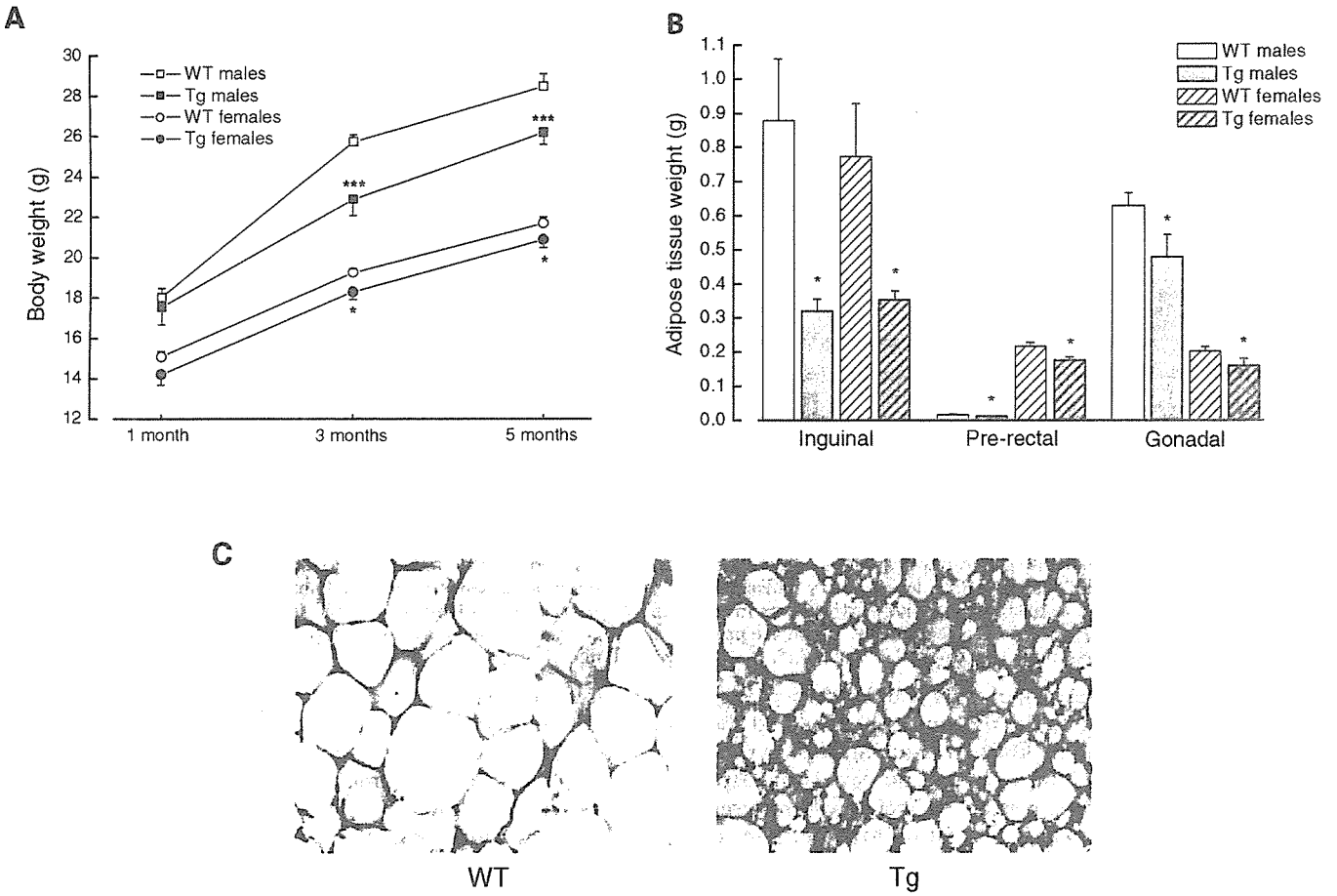

FIG. 4. Body weight, fat pad weights, and histological sections of adipose tissue in Tg mice. A, Body weight of Tg and WT mice was monitored throughout the study. B and C, Fat pad weights and HES staining of inguinal adipose tissue sections (magnification, ×500) were examined in 5-month-old mice. Values are the mean ± SEM for 10–15 mice per group (A and B) and for five mice per group (C); representative sections are shown for C. *, $P < 0.05$; ***, $P < 0.001$ *vs.* WT mice.

a higher rate of apoptosis in white fat. Immunostaining of caspase-6, a marker of apoptosis, was similar in inguinal adipose tissue sections of Tg and WT mice (not shown).

Effects of ApN on Pref-1 and C/EBPα expression in 3T3-F442A cells

We next examined whether ApN could directly modulate the expression of the transcription factors, which were modified *in vivo*. To this end, we added recombinant full-length ApN to the culture medium of differentiating 3T3-F442A adipocytes for 72 h. At the end of the experiments, we measured mRNA levels of Pref-1 and C/EBPα. When compared with control conditions, recombinant ApN doubled the abundance of Pref-1 mRNA and decreased by approximately 60% C/EBPα mRNAs (Fig. 6).

Pref-1 and C/EBPα expression in white fat of ApN-deficient mice

We tested *a contrario* the hypothesis that ApN modulates the expression of adipose growth/transcription factors and

TABLE 3. Food consumption and indirect calorimetry in Tg mice

	Males		Females	
	WT	Tg	WT	Tg
Food consumption and efficiency				
Food intake (kcal/d)	12.2 ± 0.3	11.9 ± 0.3	10.8 ± 0.3	11.9 ± 0.4[a]
Feed efficiency (g gained/kcal consumed) × 100	123.9 ± 2.9	108.5 ± 3[b]	87.0 ± 2.5	74.6 ± 2.2[b]
Energy expenditure				
Energy expenditure (kcal/g/d)	7573 ± 28	7893 ± 26[c]	ND	ND

Food intake was measured daily over a 3-month period (from 1 month up to 4 months of age). Twenty-four-hour energy expenditure was measured by indirect calorimetry in 5-month-old males. Results are the mean ± SEM for 15 mice for food consumption and eight mice for indirect calorimetry. ND, Not done.

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ *vs.* WT mice.

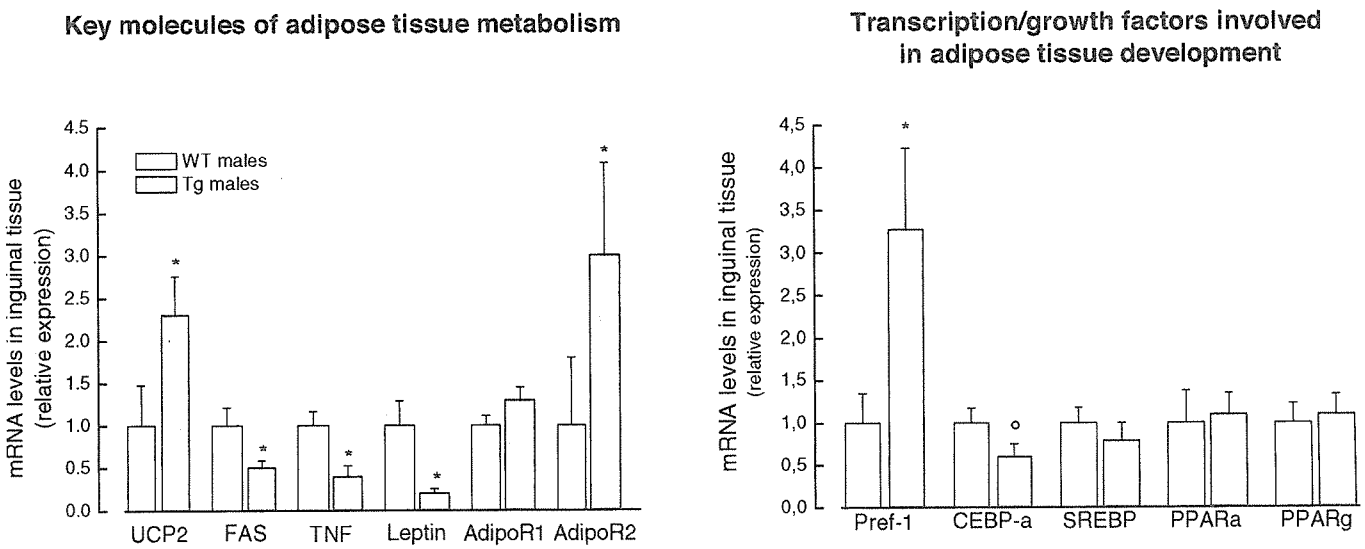

FIG. 5. Expression of several genes implicated in adipose tissue metabolism and development of Tg mice. Inguinal fat was sampled in 5-month-old in WT and Tg mice. mRNA levels of key molecules playing a role in adipose tissue metabolism (UCP 2, FAS, TNFα, leptin, and AdipoR1 and R2) as well as of transcription/growth factors involved in adipose tissue development (Pref-1, C/EBPα, SREBP-1, PPAR α and γ) were quantified by RTQ-PCR as described in Fig. 1, and are presented as relative expression compared with respective WT values. Results are the mean ± SEM for eight mice per group. °, $P = 0.06$; *, $P < 0.05$ *vs.* WT mice.

examined whether the lack of ApN could induce changes in their abundance *in vivo*. To this end, we measured the expression of Pref-1 and C/EBPα in inguinal adipose tissue of 5-month-old ApN$^{-/-}$ mice, where no circulating ApN is detectable (26). The expression of Pref-1 was reduced by approximately 80% in inguinal fat of ApN-deficient mice, whereas mRNA levels of C/EBPα were increased by approximately 50% (Fig. 7).

Discussion

Here, we generated a Tg mouse model allowing persistent and moderate overexpression of native full-length ApN targeted to WAT. Adipose mass and adipocyte size of Tg mice were reduced despite preserved calorie intake. This resulted from increased energy expenditure and up-regulation of UCP, and from abrogation of the adipocyte differentiation program, as shown by the stimulation of Pref-1 and the down-regulation of C/EBPα. We further found that recombinant ApN stimulated Pref-1 mRNA and attenuated C/EBPα expression in 3T3-F442A cells, whereas opposite changes in the expression of these genes were observed in white fat of ApN-deficient mice. Thus, besides enhanced energy expenditure, our work shows that impairment of adipocyte differentiation contributes to the antiobesity effect of ApN.

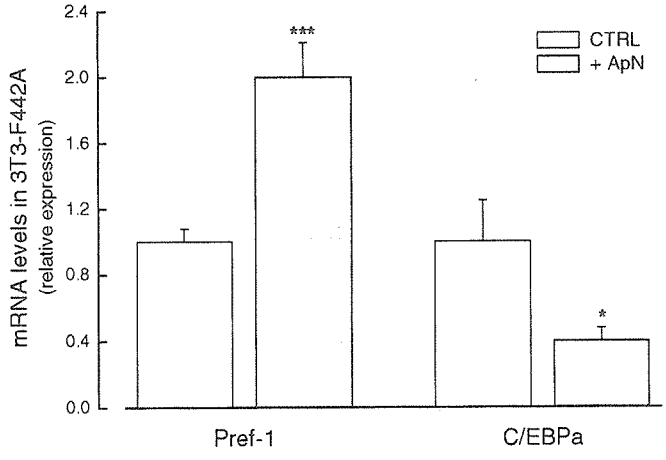

FIG. 6. Expression of Pref-1 and C/EBPα in 3T3-F442A. Differentiating adipocytes were cultured in the presence or in the absence of mouse recombinant ApN (full-length form; 1 μg/ml) for 3 d. mRNA levels of Pref-1 and C/EBPα were quantified by RTQ-PCR and are presented as relative expression compared with control values (*i.e.* in the absence of ApN). Results are the mean ± SEM for 12 experiments obtained from three independent cultures. *, $P < 0.05$; ***, $P < 0.001$ *vs.* respective controls.

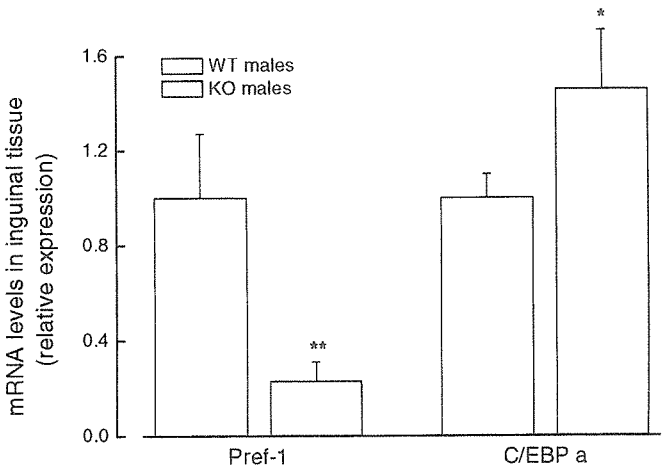

FIG. 7. Expression of Pref-1 and C/EBPα in white fat of ApN-deficient mice. Inguinal fat was sampled in 5-month-old WT and ApN-deficient mice. mRNA levels of Pref-1 and C/EBPα were quantified by RTQ-PCR and are presented as relative expression compared with WT values. Results are the mean ± SEM for six mice per group. *, $P < 0.05$; **, $P < 0.01$ *vs.* WT mice.

The average 2-fold level of ApN overexpression in WAT of aP2-ApN Tg mice most likely represents an increase that is physiologically relevant (37). This was accompanied by a similar increase of circulating ApN levels in males but by a higher increment in females. This higher increment was only observed in females fed the high-sucrose (not high-fat) diet and may actually reflect a paradoxical and diet-specific decrease of plasma ApN in control females rather than marked ApN elevation in Tg ones (this study and data not shown). Importantly, there was no change in circulating ApN multimer distribution in plasma of Tg mice compared with respective controls.

Chronic effects of ApN on body weight and adipose mass are still debated. Tg mouse models overexpressing ApN could be useful tools to address this issue. Two models have been generated so far (21, 23). In the first one, full-length ApN cDNA was mutated then placed under control of the aP2 (21); in the second one, gApN cDNA was placed under control of a liver promoter (23). In the first case, an unusual obese phenotype with bilateral exophthalmia and expansion of interscapular tissue was observed (21). In the latter case, effects on body weight were variable (unchanged or decreased), but circulating gApN was very high and influenced primarily other organs than adipose tissue (23). Our present study in Tg mice with moderate overexpression of native ApN targeted to white fat unambiguously shows that chronic and early ApN reduced fat mass and adipocyte size. These data fit *a contrario* with those obtained in our mouse lines carrying a low copy number of the same transgene, but exhibiting a decrease in ApN parameters (due to a negative feedback on endogenous production) (see introductory text) (22). The phenotype of these mice was characteristic of partial ApN deficiency (moderate increase in adiposity and insulin resistance). It is still unclear why $ApN^{-/-}$ mice, with a complete lack of ApN only exhibited insulin resistance and not obesity in response to a high-fat diet (26, 38). One may raise the possibility that, in case of complete loss of ApN, some compensatory mechanisms may operate and actually mask the effects on adipose tissue. The data of the present study also directly fit with other reports on acute or short-term effects of ApN administration (2, 16, 17).

The mechanisms underlying reduction of body fat in response to ApN supplementation are also disputed and have been ascribed either to increased energy expenditure (16, 17) or to decreased food intake (39). Here, we clearly show that reduced adiposity was not due to anorexia but rather to enhanced energy expenditure as illustrated by decreased feed efficiency and indirect calorimetric measurements. Accordingly, the expression of UCPs, molecules involved in energy dissipation (UCP2 and possibly UCP1 in this study) was augmented, in agreement with other acute or short-term reports on ApN administration (12, 16, 17).

In addition to increased energy expenditure, impaired adipocyte differentiation (34, 40) is a novel mechanism, which could contribute to reduce fat mass. Forced expression of ApN to white fat resulted in smaller (younger) adipocytes, whereas down-regulating some adipocyte markers such FAS, leptin, and TNFα. The decreased expression of the key lipogenic enzyme, FAS may play a role in the lower lipid accumulation within adipocytes. This down-regulation was associated with altered expression of transcription/growth factors known to modulate adipocyte differentiation. Thus, the preadipocyte marker, Pref-1, an epidermal growth factor, which functions in the maintenance of the preadipose state by inhibiting differentiation into adipocytes was elevated in fat tissue of Tg mice (41). Pref-1-deficient mice displayed obesity and increased serum lipid parameters (42), whereas mice overexpressing the soluble form of this growth factor showed decreased adipose tissue mass and adipocyte marker expression (41). On the other hand, the expression of the pleiotropic transcriptional activator, C/EBPα that coordinately stimulates the expression of the adipocyte genes giving rise to the adipocyte phenotype (35), was down-regulated in fat of our Tg animals. Taken together, these results suggest that adipocyte differentiation was impaired in Tg mice possibly due to altered expression of two key transcription/growth factors that orchestrate this stage. We next provided evidence that recombinant ApN induced direct changes of these key regulators in 3T3-F442A cells. Thus, at variance with another report on 3T3-L1 cells infected by a recombinant lentivirus that produced very high concentrations of the adipokine (20), our *in vitro* results support the hypothesis that ApN directly prevents the appearance of the adipocyte phenotype. Eventually, we showed that opposite modifications of Pref-1 and C/EBPα mRNA occurred in adipose tissue of ApN-deficient mice. Hence, targeting ApN to adipocyte may mitigate lipid accumulation and alter phenotype of adipocyte. This alteration of the adipocyte phenotype suggests molecular targets for the treatment of morbid obesity (43).

ApN causes adipose tissue remodeling and leads to a higher density of smaller adipocytes. These smaller adipocytes may further contribute to the metabolic action of the adipokine. Indeed, smaller adipocytes are known to better retain free fatty acids than larger ones and to release fewer inflammatory cytokines, such as TNFα that impair insulin signaling (44, 45). These cells may therefore play a role in the increased insulin sensitivity and in the hypolipemic effect of ApN.

ApN exerts its metabolic effects via two types of receptors: AdipoR1 and AdipoR2. Both types are expressed in adipocytes (5). AdipoR2 expression was up-regulated in fat of Tg mice. Because ApN may directly inhibit AdipoR2 in cultured adipocytes (20, 22), the up-regulation of this receptor isoform was rather explained by an indirect mechanism such as attenuation of TNFα expression. Indeed, TNFα mRNA was reduced in adipose tissue of Tg mice, a finding *a contrario* consonant with the enhanced expression of this cytokine observed in fat of mice with partial or complete ApN deficiency (22, 26). Taken together, these data reinforce the suggestion that ApN and TNFα exert negative reciprocal interactions on their local production in adipose tissue (26). Because TNFα has been found to decrease AdipoR2 mRNA in primary cultures of pig adipocytes (46), the local reduction of TNFα may explain why AdipoR2 mRNA was up-regulated in fat of our Tg mice. Thus, despite ApN excess, there was no down-regulation, but rather an up-regulation of ApN receptor expression in these mice. This suggests that the potency of ApN produced by these Tg mice was preserved and even possibly enhanced.

In conclusion, mice overexpressing ApN specifically in white fat showed a clear reduction in adiposity due to increased energy expenditure and to impaired adipocyte differentiation. Adipose tissue remodeling results in smaller and less mature adipocytes characterized by altered expression of lipogenic enzymes and adipocyte markers, and increased expression of UCPs and a preadipocyte marker. This prevention of the adipocyte phenotype could theoretically induce a lower rate of relapse after conventional treatment of obesity by caloric restriction (43). It also suggests molecular targets for pharmacologic treatment of morbid obesity.

Acknowledgments

We are grateful to Pr. C. Remacle (University of Louvain) for discussion, to the Tg facilities provided by the University of Louvain, to C. Diepart (University of Louvain) for skillful assistance, and to L. Montbrun (Paul Sabatier University) from the Physiopathological Exploration platform of the Toulouse Genopole for performing the calorimetric experiments.

Received June 20, 2006. Accepted December 27, 2006.

Address all correspondence and requests for reprints to: S. M. Brichard, Unité d'Endocrinologie et Métabolisme, University of Louvain/Endocrinology 5530, Avenue Hippocrate, 55, B-1200 Brussels, Belgium. E-mail: brichard@endo.ucl.ac.be.

This work was supported by grants from the Foundation of Scientific and Medical Research (3.4580.05), from the Fonds National de la Recherche Scientifique (1.5.189.04), the Fund for Scientific Development (University of Louvain), and Grant Action de recherche concertée 05/10-328 from the General Division of Scientific Research. I.B.B. has a fellowship from the Fonds pour la formation à la recherche dans l'industrie et dans l'agriculture (Belgium).

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Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

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: $13.36\pm4.25\times10^7$ arbitrary units for APN-KO and $6.74\pm2.87\times10^7$ 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 atherothrombosis, 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 adipocytokines, including tumor necrosis factor (TNF) α, leptin, resistin, and plasminogen activator inhibitor type-1.[2–4] Dysregulated production of adipocytokines participates in the development of obesity-related metabolic and vascular diseases.[2–4]

Adiponectin is an adipocytokine identified in the human adipose tissue cDNA library, and Acrp30/AdipoQ is the mouse counterpart of adiponectin (reviewed in reference[5]). 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.[5] Plasma adiponectin levels decrease in obesity, type 2 diabetes, and patients with coronary artery disease (CAD).[5–9] 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.

Original received August 4, 2005; final version accepted October 24, 2005.

From the Departments of Hematology and Oncology (H. Kato, H. Kashiwagi, M.S., S.T., T.K., H.U., Y.T., Y.Ka.) and Internal Medicine and Molecular Science (N.M., T.F., I.S.), Graduate School of Medicine, Osaka University, Suita; National Cardiovascular Center Research Institute (S.H.), Suita, Osaka; Division of Transfusion Medicine (S.M.), National Cardiovascular Center, Suita, Osaka; Department of Nutrition Management (Y.I.), Faculty of Health Science, Hyogo University, Kakogawa, Hyogo; Laboratory of Physiology, Faculty of Nutrition (J.Y.) and High Technology Research Centre (J.Y.), Kobe Gakuin University, Kobe; and Department of Blood Transfusion (Y.Ku.), Osaka University Hospital, Suita, Japan.

Correspondence to Yoshiaki Tomiyama, Osaka University, Department of Hematology and Oncology, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail yoshi@hp-blood.med.osaka-u.ac.jp

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000194076.84568.81

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 $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ (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 (1×10^8) 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 prepared as described previously.[11,17]

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.[18] For the amplification of transcripts of mouse adiponectin receptors AdipoR1 and AdipoR2, the following primers were used: mouse AdipoR1 5′-ACGTTGGAGAGTCATCCCGTAT-3′ (sense) and 5′-CTCTGTGTGGATGCGGAAGAT-3′ (antisense) and mouse AdipoR2 5′-TGCGCACACATTTCAGTCTCCT-3′ (sense) and 5′-TTCTATGATCCCCAAAAGTGTGC-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′-CTTCTACTGCTCCCCACAGC-3′ (sense) and 5′-GACAAAGCCCTCAGCGATAG-3′ (antisense) human AdipoR2 5′-GGACCGAGCAAAAGACTCAG-3′ (sense) and 5′-CACCCAGAGGCTGCTACTTC-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 *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

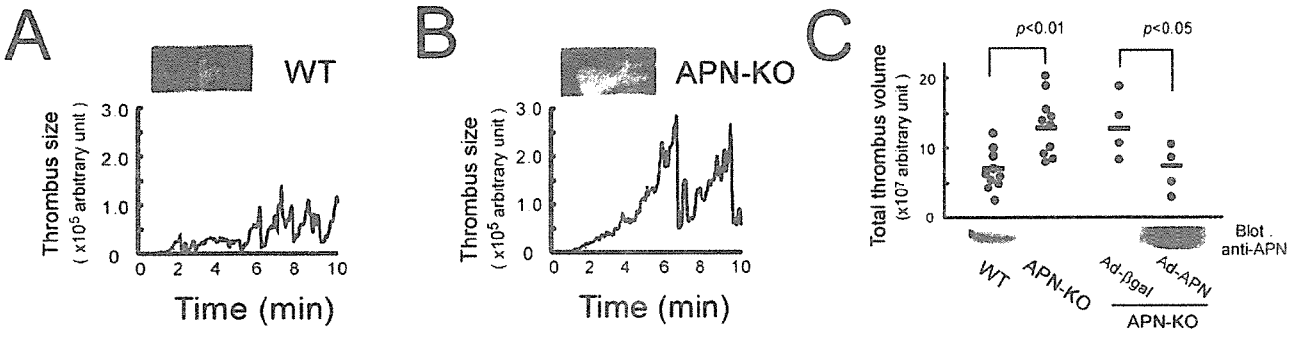

Figure 1. He-Ne laser–induced thrombus formation and adenovirus-mediated supplementation of adiponectin. Anesthetized mice were injected with Evans blue dye followed by irradiation with the He-Ne laser at the exposed left carotid artery. The representative time course of thrombus formation in (A) WT or (B) APN-KO mice is shown. (C) The total thrombus volume was significantly larger in APN-KO mice (n=10; $P<0.01$). In another set of experiments, administration of adenovirus-producing mouse adiponectin (Ad-APN) significantly attenuated the total thrombus volume, as compared with control adenovirus (Ad-βgal)-infected APN-KO mice (n=4; $P<0.05$). Plasma adiponectin levels detected in immunoblots are shown in the lower panel.

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±25.0 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±2.3 in WT mice. In APN-KO mice, there was no significant difference in the initiation time for thrombus formation (54.8±8.9 s; n=10; $P=0.46$). However, the cycles of thrombus formation during the 10-minute observation period were significantly fewer (5.4±2.0; 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 ($6.74\pm2.87\times10^7$ arbitrary units in WT mice and $13.36\pm4.25\times10^7$ 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 $12.94\pm4.67\times10^7$ 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 ($6.23\pm3.09\times10^7$ 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

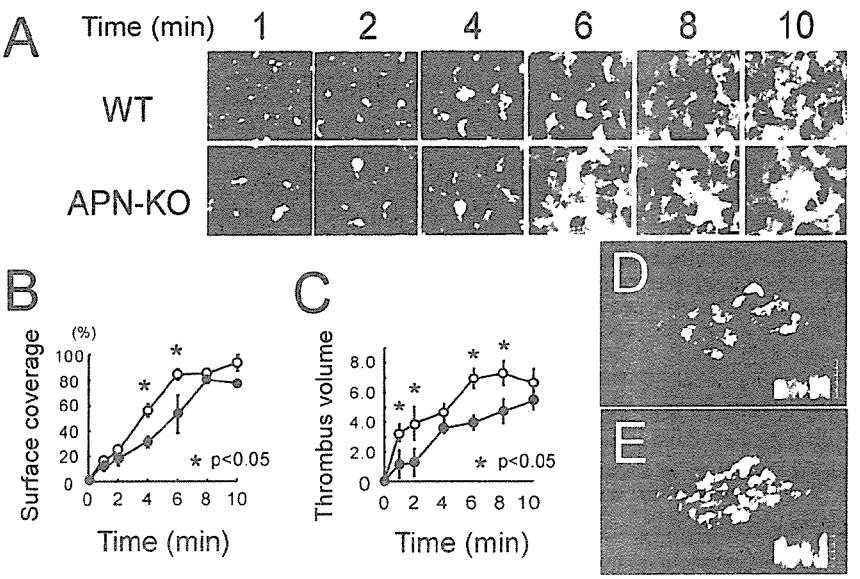

Figure 2. Thrombogenesis on a type I collagen-coated surface under flow conditions. (A) Mepacrine-labeled whole blood obtained from WT (top) or APN-KO mice (bottom) was perfused on a type I collagen-coated surface at a shear rate of 250 s^{-1}. (B) Platelet surface coverage (%) and (C) thrombus volume are shown at indicated time points. (●, WT; ○, APN-KO; *$P<0.05$). Shown are representative 3D images of thrombus formation at 6-minute perfusion in whole blood obtained from (D) WT and (E) APN-KO mice. Each inserted figure shows thrombus height.

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.[23] 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-βgal or Ad-APN to adjust platelet counts to $300\times10^3/\mu L$. 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 $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ activation and α-granule secretion at various concentrations of agonists by flow cytometry. However, neither the platelet $\alpha_{IIb}\beta_3$ 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).

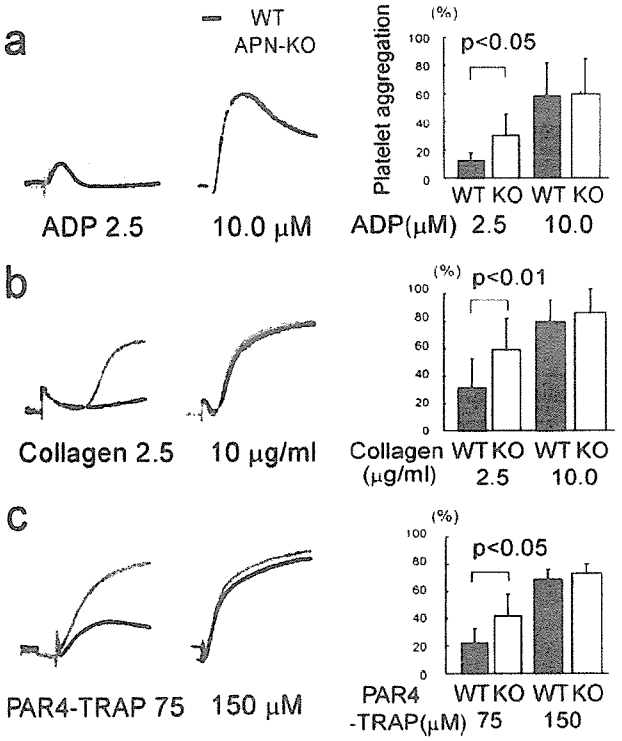

Figure 3. Enhanced platelet aggregation in APN-KO mice. Platelet aggregation in PRP obtained from WT or APN-KO mice. PRP ($300\times10^3/\mu L$) obtained from WT (black line) or APN-KO mice (gray line) was stimulated with ADP (a; n=4), collagen (b; n=4), or PAR4-TRAP (c; n=3). As compared with WT mice, platelet aggregation was enhanced in APN-KO mice at low concentrations of agonists.

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-βgal into WT mice, the plasma adiponectin levels in Ad-APN-infected mice reached ≈4 times higher than those in Ad-βgal-infected WT mice (8.5±0.6 μg/mL for Ad-βgal 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 ($4.38\pm0.75\times10^7$ arbitrary units for Ad-βgal and $2.75\pm0.61\times10^7$ arbitrary units for Ad-APN; n=7; $P<0.05$; Figure 6C).

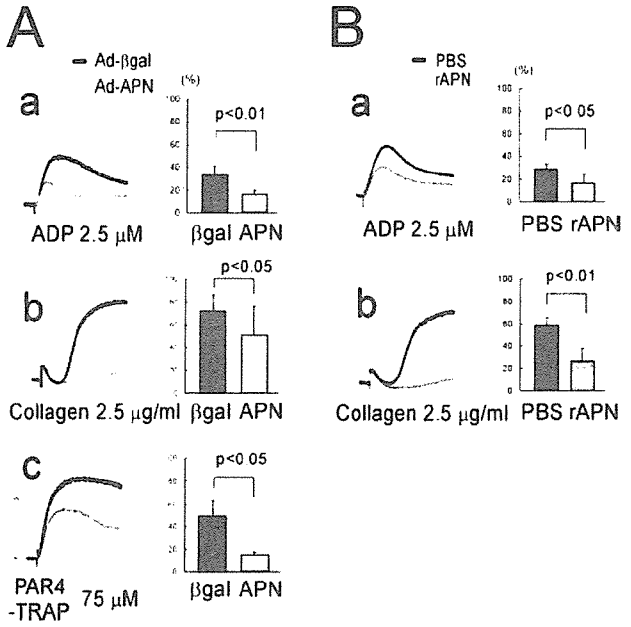

Figure 4. Effects of in vitro supplementation of adiponectin or recombinant adiponectin on the enhanced platelet aggregation in APN-KO mice. (A) One volume of PRP from APN-KO mice was mixed with ≈4 volumes of PPP from APN-KO mice injected with Ad-βgal (black line) or Ad-APN (gray line) to obtain a platelet concentration of $300\times10^3/\mu L$. Platelets were stimulated with indicated agonists (n=4). (B) Mouse recombinant adiponectin (40 μg/mL, gray line) or PBS (black line) was added to PRP from APN-KO mice. Platelets were adjusted to 300×10^3 platelets/μL and stimulated with indicated agonists (n=4).

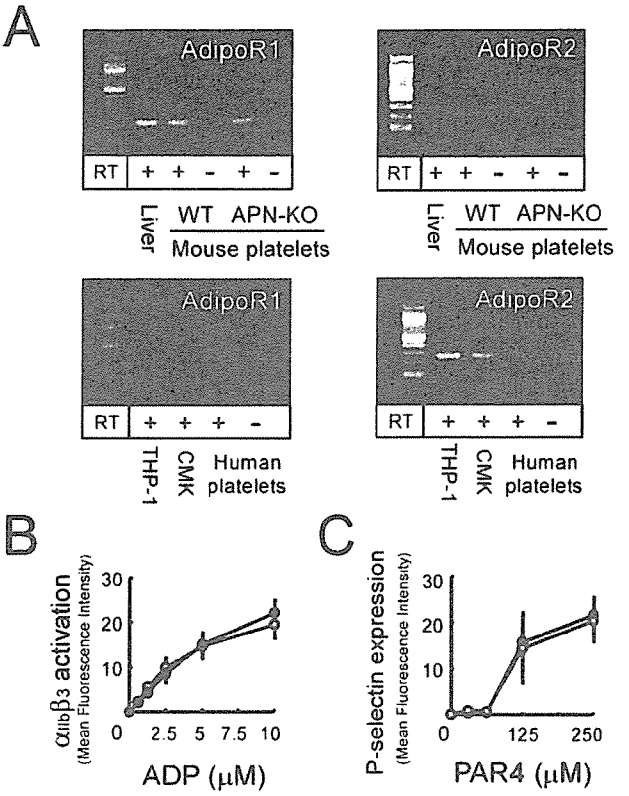

Figure 5. Expression of adiponectin receptors and effects of adiponectin deficiency on platelet function. (A, top) Expressions of transcripts for adiponectin receptors, AdipoR1 (133-bp fragments) and AdipoR2 (156-bp fragments), in platelets from WT or APN-KO mice were examined by RT-PCR. The liver was used as a positive control. (Bottom) Expressions of transcripts for adiponectin receptors, AdipoR1 (196-bp fragments) and AdipoR2 (243-bp fragments), in CMK cells, as well as human platelets, were examined by RT-PCR; 100-bp DNA Ladder (New England Biolabs) was used as a marker. Effects of adiponectin deficiency on (B) $\alpha_{IIb}\beta_3$ activation and (C) α-granule secretion. PRP obtained from WT (●) or APN-KO (○) mice in the presence of phycoerythrin-JON/A mAb or FITC-anti-P-selectin mAb was stimulated with the indicated agonist and then analyzed by flow cytometry without any washing. There were no significant differences in platelet $\alpha_{IIb}\beta_3$ activation or P-selectin expression between WT and APN-KO mice (n=4).

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 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.[8] Pischon et al[9] 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.[10,12] 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).[24] 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.[12,25] In addition, recombinant adiponectin increased NO production in vascular endothelial cells.[26] To rule out any

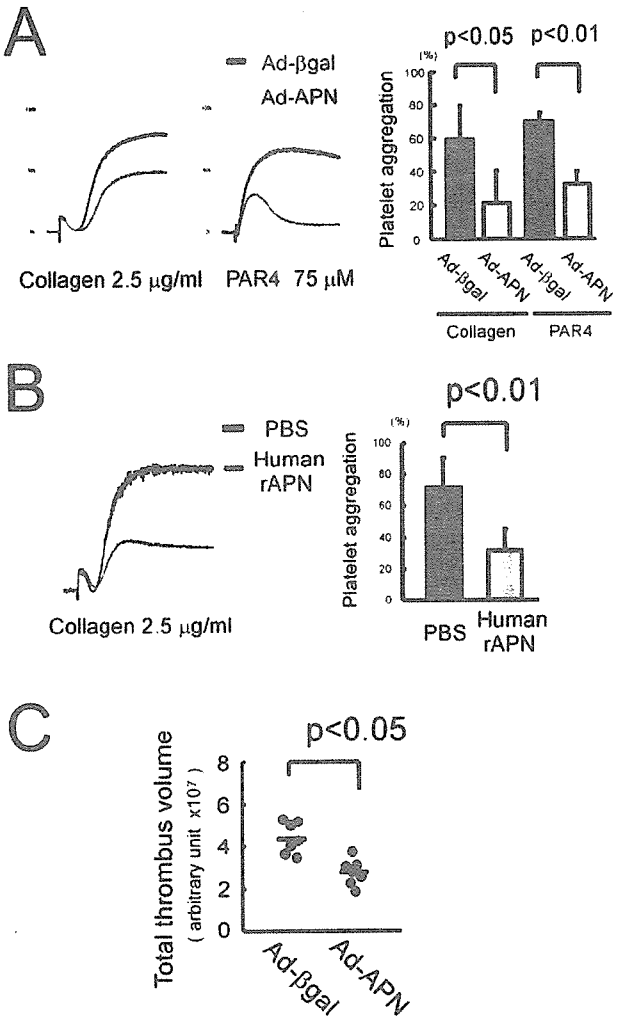

Figure 6. Overexpression of adiponectin additionally attenuates thrombus formation in WT mice. (A) Platelet aggregation in PRP obtained from WT mice injected with either Ad-βgal or Ad-APN. PRP ($300\times10^3/\mu L$) obtained from WT mice injected with either Ad-βgal (black line) or Ad-APN (gray line) was stimulated with collagen or PAR4-TRAP (n=4). Administration of Ad-APN significantly attenuated platelet aggregation in WT mice. (B) Human recombinant adiponectin (40 μg/mL, gray line) or PBS (black line) was added to PRP ($300\times10^3/\mu L$) from control subjects. Platelets were stimulated with collagen (n=7). (C) He-Ne laser–induced thrombus formation in WT mice injected with either Ad-βgal or Ad-APN. Administration of Ad-APN in WT mice additionally reduced the total thrombus volume in the carotid artery thrombus model (n=7, $P<0.05$).

effect of adiponectin on vascular cells, we examined in vitro thrombus formation on a type I collagen-coated surface under flow conditions, as well as platelet aggregation in APN-KO mice. Thus, the enhanced platelet function in APN-KO mice was still evident even in the absence of vascular cells. Moreover, human and mouse recombinant adiponectin attenuated the aggregation response obtained from control human subjects and from APN-KO mice, respectively. Thus, adiponectin inhibits platelet function. However, the mechanism by which adiponectin attenuates platelet aggregation and arterial thrombus formation in vivo remains unclear. During thrombogenesis, platelets adhere to altered vascular surfaces or exposed subendothelial matrices, such as collagen, and then become activated and aggregate to each other.[16] 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.[27] 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 α_2-macroglobulin, thrombospondin-1 (TSP-1), and several growth factors.[5,23,28] Interestingly, TSP-1, after secretion from platelet α granules, may participate in platelet aggregation by reinforcing interplatelet interactions through direct fibrinogen-TSP-fibrinogen and TSP-TSP crossbridges.[29,30] 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.

Acknowledgments

This study was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology in Japan; from the Ministry of Health, Labor, and Walfare in Japan, Astellas Foundation for Research on Metabolic Disorder, Tokyo, Japan; and Mitsubishi Pharma Research Foundation, Osaka, Japan.

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