Adipokines identified as new downstream targets for adiponectin: lessons from adiponectin-overexpressing or -deficient mice

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Ge Q, Ryken L, Noel L, Maury E, Brichard SM. Adipokines identified as new downstream targets for adiponectin: lessons from adiponectin-overexpressing or -deficient mice. Am J Physiol Endocrinol Metab 301: E326–E335, 2011. First published May 3, 2011; doi:10.1152/ajpendo.00153.2011.—Adipokines play a central role in the pathogenesis of the metabolic syndrome. Among them, adiponectin (ApN), a master regulator of immune and fuel homeostasis, is decreased. Identifying downstream adipokines targeted by ApN may help in deciphering this syndrome. We have generated transgenic mice, allowing persistent and moderate overexpression of ApN (ApN-Overex) specifically in white adipose tissue (AT). We took advantage of this model to unravel the adipokine secretion profile triggered by ApN. AT was fractionated into adipocytes and stromal-vascular cells (SVC), which were cultured for 8 h. Profiling of secretory products by antibody arrays and subsequent ELISAs showed that the secretion of three proinflammatory factors (IL-1β, IL-21, TNFα) and three hematopoietic growth factors [GF; thrombopoietin and granulocyte (macrophage) colony-stimulating-factors] was reduced in adipocytes of ApN-Overex mice compared with wild-type mice. In the SVC of these mice, besides the hematopoietic GFs, the secretion of another GF (vascular endothelial GF receptor 1), two chemokines (RANTES and ICAM-1), and two proinflammatory factors (IL-6 and IL-12p70) was reduced as well. Only one cytokine, IL-1 receptor 4, was oversecreted by SVC of ApN-Overex mice, which may exhibit anti-inflammatory properties. Most of these changes in secretion were due to corresponding changes in mRNAs. A reverse profile of adipokine expression was observed in ApN-KO mice. In conclusion, ApN regulates in vivo the secretion of downstream adipokines, thereby inducing a shift of the immune balance in both adipocytes and SVC toward a less inflammatory phenotype. These downstream adipokines may be new therapeutic targets for the management of the metabolic syndrome.

Adipose tissue (AT) secretes a number of bioactive peptides, collectively named adipokines. They may exert endocrine actions on distant organs or autocrine/paracrine actions on AT. They play a central role in energy and vascular homeostasis as well as in immunity (23). Virtually all known adipokines are dysregulated in obesity, type 2 diabetes, and metabolic syndrome, leading to overproduction of deleterious adipokines and hyposecretion of defensive ones such as adiponectin (ApN). Such a dysregulation triggers the development of a low-grade proinflammatory state, which is considered to build the common soil for the development of obesity-linked disorders (23). Resetting the immunologial balance in adipose tissue may be a crucial approach for the future management of the metabolic syndrome. Adipokines may be potential therapeutic targets.

ApN has emerged as a master regulator of immune/inflammatory and fuel homeostasis (14). Besides its vascular protective action (32), ApN exerts anti-inflammatory effects on other organs such as liver, colon, cardiac, or skeletal muscles (2, 18, 30, 37). As yet, the autocrine and paracrine anti-inflammatory effects of ApN on its own production site have been scarcely described (1, 9, 22). Moreover, its effects on AT secretory functions have been reported in only a few in vitro studies (1, 9). Thus, identifying downstream adipokines targeted by ApN in vivo may be instrumental in highlighting its action on AT.

We have generated a transgenic mouse model, allowing persistent and moderate overexpression of ApN (ApN-Overex) specifically in white AT by using the aP2 promoter (3). We took advantage of this unique in vivo model to unravel the adipokine secretion profile triggered by ApN in AT. We also examined whether a reverse profile occurred in ApN-knockout (ApN-KO) mice (22).

RESEARCH DESIGN AND METHODS

Animals. Male ApN-Overex mice and their wild-type (WT) littermates were housed in groups of two to four at a constant temperature (22°C) with a fixed 12:12-h light-dark cycle. The two groups of mice were matched for weaning body weight. They were weaned to a high-sucrose diet (TD00220; Harlan, Horst, The Netherlands), since mouse phenotype and degree of transgene expression were characterized in those conditions (3). In an additional experiment, male ApN-KO mice, which exhibit a complete lack of ApN in fat and plasma, and their WT controls (mice of the same genetic background that were raised together with ApN-KO mice but were not their littermates) were also used. These animals were housed in the same conditions as ApN-Overex mice and received the same diet from weaning.

Body weight was measured every week. At the end of the experiments, mice were euthanized by decapitation (between 0800 and 1000). Tail vein blood samples were saved before euthanization. Inguinal fat pads (the AT site where the effects of the transgene ApN were the most pronounced) (3) were quickly removed, weighed, and frozen in liquid nitrogen and stored at –80°C for subsequent experiments.

The University of Louvain Animal Care Committee approved all procedures.

Quantification of circulating parameters. Blood glucose was measured using a glucometer (MediSense Precision Xtra Plus; Abott-Medisense, Louvain-la-Neuve, Belgium). Plasma insulin was measured by RIA (kit from Linco Research, St. Charles, MO) and plasma triglycerides by a colorimetric method (kit from BD Biosciences, Erembodegem, Belgium).

AT immunohistochemistry and morphometry. AT samples were fixed in 10% formaldehyde for 24 h and embedded in paraffin. Five-micrometer-thick sections were processed using a rat monoconal antibody directed against mouse F4/80, a macrophage marker (AbD; Serotec, Dusseldorf, Germany), according to the manufacturer’s recommendations. Sections were pretreated in a microwave oven in Tris-citrate buffer (pH 6.5) for one cycle of 3 min at 750 W.
and three cycles of 3.5 min at 350 W. Binding of the antibody was detected by applying a second antibody to 30 min at room temperature, which was a rabbit anti-rat immunoglobulin conjugated to peroxidase-labeled polymer (En Vision +; Dako, Copenhagen, Denmark). Peroxidase activity was revealed with 3,3’-diaminobenzidine substrate (Dako), which produces a brown staining. For quantification, the number of immunolabeled macrophages per microscopical field (density) was counted at magnification 400. The index of insulin sensitivity is calculated as G/I (mg/ng) (38). *P < 0.05, **P < 0.01 vs. WT. Columns with no values represent mice that were not measured.

Table 1. Gene sequences used as forward and reverse primers for RT-quantitative PCR

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<td>CCCTTGAOCTCTCTGTATTCC</td>
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<td>AdipoR1</td>
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<td>GCTGACCTCCAGGATTCT</td>
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<td>CD11c</td>
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<td>GCSF</td>
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ApN, adiponectin; AdipoR1, and -2, ApN receptor 1 and 2, respectively; Mgl1, macrophage galactose N-acetyl-galactosamine-specific lectin 1; GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; RANTES, regulated upon activation, normal T cell expressed, and secreted; TPO, thrombopoietin; VEGFR1, VEGF receptor 1.

Briefly, fresh inguinal adipose tissue (0.3 g) was finely minced and incubated in a shaking water bath at 37°C in 750 μl of Krebs-BSA buffer (KRAB) containing collagenase A (2.5 mg/0.3 g tissue; Roche Diagnostics, Vilvoorde, Belgium) for 15 min. The digested tissue was filtered through a metallic mesh and then centrifuged at 400 g for 1 min. The supernatant (containing the adipocytes) and pellet (containing the stromal-vascular cells (SVC)) were separated and washed three times in KRAB. The pellet (SVC) was resuspended in 80 μl of KRAB. Isolated cells were then cultured in 48-well plates. Experiments were performed in duplicate. Eighty microliters of packed adipocytes or 40 μl of SVC was distributed per well, which contained 700 μl of MEM supplemented with 1% BSA and 1:500 (vol/vol) antibiotics (Primocin; InvivoGen). Cells were allowed to stabilize for 1 h in this medium. Next, the medium was renewed, and the cells were cultured for 8 h. After culturing, aliquots of medium were stored at −20°C, and cells were harvested and stored at −80°C. DNA content was measured in cell samples, as described before (25).

Table 2. Characteristics of ApN-overexpressing mice

<table>
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<th>8 Wk</th>
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<th>12 Wk</th>
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<tr>
<td>WT</td>
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<td>22.3</td>
<td>25.1</td>
</tr>
<tr>
<td>ApN-Overex</td>
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<td>22.3</td>
<td>25.1</td>
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<td><strong>Inguinal adipose tissue</strong></td>
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<tr>
<td>Fat pad weight, mg</td>
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<td>387</td>
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<td>Relative weight, %body wt</td>
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<td>15.4</td>
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<td>Adipocyte size, μm²</td>
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<td>74.2</td>
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<td>Macrophage density</td>
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<td>Plasma triglycerides, mg/dl</td>
<td>48.3</td>
<td>43.3</td>
<td>58.9</td>
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<tr>
<td>Blood glucose, mg/dl</td>
<td>156</td>
<td>149</td>
<td>164</td>
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<tr>
<td>Plasma insulin, ng/ml</td>
<td>0.56</td>
<td>0.06</td>
<td>0.59</td>
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<tr>
<td>Insulin sensitivity (G/I), mg/ng</td>
<td>2.90</td>
<td>2.06</td>
<td>2.88</td>
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Data are means ± SE for 5–6 mice/group. WT, wild type; ApN-Overex, overexpression of adiponectin; G/I, glucose/insulin. ApN-Overex mice and their WT littermates were studied at the age of 8, 10, and 12 wk. Blood was sampled at 8000–10000. Organ weight refers to pairs of inguinal fat pads, and relative organ weight is expressed as %body weight (i.e. as g/g body wt × 100). Adipocyte size is estimated from mean adipocyte area. Macrophage density corresponds to mean macrophage number per microscopical field at magnification ×400. The index of insulin sensitivity is calculated as G/I (mg/ng) (38). * P < 0.05, **P < 0.01 vs. WT. Columns with no values represent mice that were not measured.
Cytokine antibody arrays and ELISAs or RIA on adipocyte- or SVC-conditioned medium. Screening for cytokines secreted by cultured adipocytes or SVC was performed by hybridizing medium with antibody-coated membranes according to the protocol supplied by the manufacturer [RayBio Mouse Cytokine Antibody Array C Series 2000, a kit combining membranes of Arrays III, VI, and V and allowing the simultaneous detection of 144 cytokines (cat. no. AAM-CYT-2000, for details see http://www.raybiotech.com/manual/ Antibody%20Array/AAM-CYT-2000.pdf; RayBiotech)]. Briefly, 1 ml of medium was incubated with arrayed antibody supports for 1 h and 30 min at room temperature; membranes were then washed and incubated with the mix of biotin-conjugated antibodies for another 1 h and 30 min. After washing, horseradish peroxidase-conjugated streptavidin was added to the membranes for 1 h at room temperature. Spot intensities on membranes were quantified by scanning densitometry (Gel-Doc2000; Bio-Rad Laboratories) and analyzed with Quantity One (Bio-Rad Laboratories). Nonconditioned media (containing 1% BSA) were used as negative controls, as described already (25). Signals were normalized to internal positive controls present on each membrane (see Fig. 2) and then expressed as intensity units per micrograms DNA in each cellular fraction. The experiments on both genotypes of mice were always carried out simultaneously.

The cytokines identified by protein antibody arrays as differentially secreted between the two genotypes of mice (P < 0.1) were further quantified by specific ELISAs: chemokine (C-C motif) ligand 5 (LIX), granulocyte colony-stimulating factor (GCSF), granulocyte macrophage colony-stimulating factor (GM-CSF), growth arrest-specific 6, IL-6, IL-21, IL-17B, IL-12p70, IL-1 receptor 4 (IL-1R4/ST2), intercellular adhesion molecule-1 (ICAM-1), regulated upon activation, normal T cell expressed and secreted (RANTES), TNFα, thrombopoietin (TPO), and VEGF receptor 1 (VEGFR1) (all from RayBiotech, except for IL-12p70 and IL-1R4, which were from R & D Systems Europe, Abingdon, UK). However, we did not quantitate tissue inhibitor of metalloproteinases-2 by this method, because its function on AT is not clear, and there was no commercial kit available. On the other hand, we measured TNFα by ELISA, which actually escaped detection by arrays, because of the known relationships between TNFα and ApN (22) and our past experience [the background generated by nonconditioned medium may mask the low amounts of some adipokines secreted by cultured cells (such as TNFα)] (25).

ApN was measured in medium by a RIA kit (Linco Research).

RNA extraction and real-time quantitative polymerase chain reaction. Total RNA from cells or tissue was extracted by using TriPure Isolation Reagent (Roche Diagnostics, Vilvoorde, Belgium); 0.2–2 μg of total RNA were reverse transcribed as described (8). Real-time quantitative polymerase chain reaction (RTqPCR) primers were designed using Primer Express Software (Applied Biosystems; see Table 1). For VEGFR1 and IL-1R4 mRNAs, the sets of primers used did not discriminate between soluble or transmembrane receptor isoforms, and thus, global gene expression was measured. Four to forty nanograms of total RNA equivalents were amplified with iQSyber Green Supermix (Bio-Rad) containing 300 nM of each specific primer using the iCycler iQ Real-Time PCR detection System (Bio-Rad). Briefly, the threshold cycles (C_T) were measured in duplicate. ΔC_T values were calculated in every sample for each gene of interest as follows: C_T gene of interest – C_T reporter gene, with cyclophilin as the reporter gene. Relative changes in the expression level of one specific gene (ΔΔC_T) were calculated as ΔC_T of the test group minus ΔC_T of the reference group and then presented as 2-ΔΔC_T.

Western blot. Adipose tissue was homogenized in lysis buffer (Cell Signaling Technology, BIOKÉ). Leiden, The Netherlands) supplemented with 100 mM NaF and 1% protease inhibitor cocktail (Active Motif, Rixensart, Belgium). Thirty micrograms of protein was dissolved in Laemmli buffer, subjected to SDS-PAGE under reducing and heat-denaturating conditions, and then transferred to PVDF membrane. The following antibodies were used for immunodetection: anti-AMPKα, Phospho-AMPKα (Thr172), anti-p44/42 MAPK (Erk1/2), phosho-p44/42 MAPK (Erk1/2), (Thr172/Tyr204), anti-SAPK/JNK, phospo-SAPK/JNK (Thr183/Tyr185). Each antibody was used according to the manufacturer’s instructions (Cell Signaling Technology, BIOKÉ). Signals were revealed by enhanced chemiluminescence. Band intensities were quantified by scanning densitometry (Gel-Doc2000; Bio-Rad Laboratories), analyzed with Quantity One (Bio-Rad Laboratories), and normalized to actin band intensity.

NF-κB activity. The TransAM NF-κB p65 transcription factor assay kit (Active Motif, Rixensart, Belgium) was used as described already (26). Binding of p65 NF-κB transcription factors was detected by ELISA. Ten micrograms of adipose tissue proteins per well, extracted as indicated for Western blots, was used. Samples were run in duplicate.

Presentation of the results and statistical analysis. The results are means ± SE for the indicated numbers of mice. Comparisons between two different groups were carried out using two-tailed unpaired Student’s t-test. Differences were considered statistically significant at P < 0.05. For the screening by cytokine antibody arrays, the differences that were statistically significant (P < 0.05) and those that were “borderline” (P < 0.1) were considered for subsequent analysis to avoid false-negatives due to the low number of mice used in this screening.

Fig. 1. Adiponectin (ApN) production by adipocytes and stromal-vascular cells (SVC) from ApN-overexpressing (ApN-Overex) mice. Adipocytes and SVC were isolated from adipose tissue of transgenic mice and wild-type (WT) littermates and cultured independently for 8 h. A: ApN gene expression was quantified by RT-qPCR, normalized to the levels of cyclophilin, and presented as relative expression compared with WT adipocytes. B: ApN secreted in medium was measured by RIA and expressed as ng/μg DNA in each cellular fraction. Values are means ± SE for 7–8 mice/group. *P < 0.05, ***P ≤ 0.001 vs. WT mice.
RESULTS

Overexpression of ApN reduced body weight and adiposity and improved lipid and glucose parameters. Because ApN overexpression targeted to white AT resulted in reduced adiposity and improved metabolic profile (3), we first examined the evolution of these parameters. Inguinal fat pad was studied because the expression of the transgene was the most pronounced in this adipose site (3).

ApN overexpression did not influence body weight, fat pad weight, plasma triglycerides, glucose homeostasis, or insulin sensitivity in 8- and 10-wk-old mice (Table 2). However, all these parameters were significantly modified from the age of 12 wk; adiposity and circulating triglycerides were reduced, whereas insulin action was enhanced, as expected (3) (Table 2). Therefore, we used 10-wk-old mice to study the specific and direct effects of ApN on AT secretory function without the interference of any obvious confounding factors. We also checked that adipocyte size was similar in 10-wk-old ApN-Overex mice and their WT littermates by morphometric analysis of AT sections. There was also no difference in macrophage number between the two genotypes of mice, as shown by immunohistochemistry using the specific macrophage marker F4/80 (Table 2).

ApN production by adipocytes and SVC of ApN-overex mice. ApN gene was overexpressed in both cellular fractions of AT in transgenic mice compared with WT mice (Fig. 1A). However, the overall expression of ApN was much higher in adipocytes than in SVC (which contain macrophages), in line with the fact that the transgene was placed under control of aP2, which is much more abundantly (~10,000-fold) expressed in adipocytes than in macrophages (11). Accordingly, the levels of ApN secreted in culture medium were higher in ApN-Overex mice than in WT mice, with again a more abundant secretion by adipocytes (Fig. 1B).

Gene expression levels of ApN receptors (AdipoR1 and AdipoR2) were measured in both cellular fractions and did not markedly vary between mice of the two genotypes (data not shown).

Fig. 2. Cytokine antibody array membranes used to detect adipokines secreted by each cellular fraction of adipose tissue. A set of 3 Raybiotech membranes, which tests 144 cytokines, was used and probed with 8-h conditioned medium. This figure illustrates the representative arrays of adipocyte- or SVC-conditioned medium from 1 WT mouse. Highlighted adipokines represent the factors, which turned out to be differentially secreted between mice of the 2 genotypes. Each color corresponds to a specific factor family. GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; RANTES, regulated upon activation, normal T cell expressed, and secreted; LIX, chemokine (C-X-C motif) ligand 5; TPO, thrombopoietin; TIMP-2, tissue inhibitor of metalloproteinases-2; ICAM-1, intercellular adhesion molecule-1; VEGFR1, VEGF receptor 1.
Adipokine secretion profiling in ApN-Overex mice. Media from 8-h-cultured adipocytes or SVC were screened by cytokine antibody arrays (Fig. 2). Among the 144 cytokines tested, more than 40 were secreted by each cellular fraction in mice of both genotypes. The secretory profile of adipocytes showed that nine cytokines were or tended to be less secreted by ApN-Overex mice than WT mice (P < 0.1; Fig. 3A). In the stromal-vascular fraction, 10 cytokines were or tended to be differentially secreted between mice of the two genotypes (P < 0.1; Fig. 3B). These cytokines belong to five families: proinflammatory factors, growth factors, chemokines, anti-inflammatory factors, and extracellular matrix/growth arrest factors (Fig. 2).

These adipokines (i.e., differently secreted based cytokine array screening; P < 0.1 for ApN-Overex vs. WT) were further quantified by specific ELISAs. TNFα was also measured by this method. Only the adipokines that turned out to be different after ELISA quantification are shown in Fig. 4 (P < 0.05 for ApN-Overex vs. WT).

In adipocyte-conditioned media, the secretion of three pro-inflammatory factors (IL-17B, IL-21, TNFα) and three hematopoietic growth factors (TPO, GM-CSF and GCSF) was reduced in ApN-Overex mice compared with WT mice (Fig. 4A). In SVC-conditioned media of transgenic mice, besides the two hematopoietic growth factors (GM-CSF and GCSF), the secretion of VEGFR1, two chemokines (RANTES, ICAM-1), and two proinflammatory factors (IL-6, IL-12p70) was reduced as well. Only one cytokine was oversecreted by SVC of ApN-Overex mice, IL-1R4 that exhibits anti-inflammatory properties (Fig. 4B).

Gene expression of adipokines in adipocytes and SVC of ApN-Overex mice. To investigate whether the adipokine secretion changes were in part mediated by pretranslational mechanisms, we quantified gene expression. Most adipokines exhibited a similar pattern between mRNA abundance and secretion levels. Thus, in adipocytes, mRNA levels of most adipokines shown in Fig. 4 were downregulated in ApN-Overex mice (Fig. 5A). In SVC, mRNA levels of most adipokines were downregulated as well, whereas IL-1R4 mRNA was upregulated (Fig. 5B). The attenuated inflammatory response of SVC is consistent with a phenotypic switch of macrophages (4). Thus, in SVC, the abundance of an M1 marker, CD11c, which characterizes a proinflammatory macrophage phenotype, was halved in ApN-Overex mice (relative expression to WT: 0.56 ± 0.09 vs. 1.00 ± 0.13; n = 6/group, P < 0.05), whereas the mRNA abundance of an M2 marker, macrophage galactose N-acetyl-galactosamine-specific lectin-1, which characterizes an anti-inflammatory phenotype, was increased twofold (2.16 ± 0.46 vs. 1.00 ± 0.26; n = 6/group, P < 0.05), in line with the opposite phenotype reported in ApN-KO mice (31).

Fig. 3. Screening secretome of adipocytes and SVC from ApN-Overex mice. Adipocytes and SVC were cultured independently for 8 h. Conditioned media were incubated with the sets of cytokine antibody arrays like those shown in Fig. 2. Adipokine levels were measured by chemiluminescence, normalized to internal positive controls, and expressed as intensity units (INT)/μg DNA in each cellular fraction. Only the adipokines, which differed (or tended to differ) between the 2 genotypes of mice, are shown in the histograms. A: adipocyte secretome screening: 9 adipokines were or tended to be less secreted by ApN-Overex mice than WT mice. B: SVC secretome screening: 10 adipokines were or tended to be differentially secreted between ApN-Overex and WT mice. Values are means ± SE for 4 mice/group. *P < 0.05, **P < 0.01 vs. WT mice. Differences, which tended to be but were not statistically significant, are also indicated in this figure (#P < 0.1) and were considered for subsequent ELISA analysis. Gas-6, growth arrest-specific 6.

Gene expression of adipokines in adipocytes and SVC of ApN-KO mice. We next explored whether ApN was the specific regulator of these pretranslational changes and measured mRNA levels of these cytokines in ApN-deficient mice of the same age and receiving the same diet. It should be noted that, at this age, there were also no differences in body weight, fat pad weight, circulating glucose, or triglyceride levels between ApN-KO mice and their WT controls (data not shown). In adipocytes, TPO mRNA was increased in ApN-KO mice (P < 0.01 vs. WT; Fig. 6A) and thus showed a reverse pattern of expression compared with ApN-Overex mice. In SVC, gene expression of IL-12p70, ICAM-1, and VEGFR1 was increased in ApN-KO mice, whereas IL-1R4 mRNA was decreased as expected (Fig. 6B). Thus, some adipokines displayed a reverse gene expression profile in ApN-KO mice compared with ApN-Overex mice.

NF-κB, JNK, ERK1/2, and AMP-activated protein kinase activity in adipose tissue of ApN-Overex mice. Several molecular pathways, particularly those involving activation of nuclear factor κ-light-chain enhancer of activated B cells (NF-κB), JNK, and ERK1/2, may play crucial roles in linking obesity to low-grade inflammation (16, 17, 39). In addition, AMP-activated protein kinase (AMPK) is a well-established downstream event of ApN in AT (41). Therefore, we investi-
gated these pathways to unravel the mechanisms underlying the anti-inflammatory properties of ApN.

Gene expression of IκBα, an endogenous inhibitor of NF-κB activity, was increased approximately twofold ($P < 0.001$) in adipose tissue of ApN-Overex mice compared with WT mice, and this was accompanied by an ~20% decrease in NF-κB activity ($P < 0.01$ vs WT) (Fig. 7A). ERK1/2 phosphorylation was also reduced by ~35% in ApN-Overex mice (Fig. 7B). However, neither JNK protein nor JNK phosphorylation was affected by ApN (data not shown). Interestingly, both AMPK protein expression and phosphorylation were increased in AT of ApN-Overex mice by 60 and 40%, respectively ($P < 0.05$ for both; Fig. 7C). Thus, the anti-inflammatory effect of ApN on AT was associated with reduction of NF-κB and ERK1/2 and activation of AMPK.

**DISCUSSION**

The aim of this study was to investigate in vivo the effects of ApN on AT secretory function before the emergence of confounding factors. We used a unique transgenic mouse model generated in our laboratory, which exhibits a moderate overexpression of native full-length ApN targeted to white AT with no changes in the distribution of ApN multimers (3).

Five-month-old transgenic mice were characterized by reduced adiposity and adipocyte size. The mechanisms responsible for this phenotype involved increased energy expenditure and altered adipocyte differentiation (3). These mice also showed improved insulin sensitivity and lipid profile when challenged by a high-sucrose diet (3). As yet, the in vivo molecular modifications triggered by ApN before any changes in adiposity, insulin action, and circulating lipids are still unravelled in AT. Therefore, we used 10-wk-old mice to study the direct and specific effect of ApN on AT secretory function. We also used ApN-KO mice of the same age, which similarly showed no difference in body weight, fat mass, or metabolic parameters compared with WT controls.

Autocrine/paracrine effects of ApN on AT secretion have been reported in vitro by some groups. ApN attenuated LPS-induced IL-6 and TNFα release in primary pig adipocytes (1). The globular domain of ApN also reduced the secretion of several cytokines, as shown by protein array analysis of culture medium from human mammary adipocytes differentiated in vitro (9). However, these in vitro effects of ApN could not completely reflect the actual in vivo conditions and merely focused on one cellular fraction of AT. Herein, we show that ApN downregulated the secretion of six to seven cytokines in
both cellular fractions of AT and upregulated one cytokine in the SVC fraction ex vivo. Alternatively, ApN may have prevented the immune deregulation that occurred with increasing adiposity and aging. It should also be stressed that the lower secretion of adipokines by transgenic mice was not explained by either a reduced yield of cell number (since data were actually normalized per DNA) or a reduction of fat cell size, because adipocyte size was actually similar in transgenic and WT mice.

By using cytokine antibody array and ELISA approaches, in addition to already known adipokines, we identified several adipokines as newly secreted by AT and/or regulated by ApN. Besides decreasing TNFα and IL-6, whose role in propagating inflammation and inducing insulin resistance is well known (23), ApN downregulated (or prevented the stimulation of) three other proinflammatory factors (i.e., 3 interleukins: IL-12, IL-17B, and IL-21) whose function on or presence in AT is still poorly documented. Yet these three interleukins play a role in initiating and shaping immune-inflammatory responses (6, 29, 43). IL-12 is also thought to contribute to the development of atherosclerosis, and its circulating levels were increased in patients with type 2 diabetes (40). The chemokines ICAM-1 and RANTES, as well as the granulocyte (macrophage) growth factors (GCSF and GM-CSF) are implicated in adipose tissue inflammation/immunity by recruiting monocytes/macrophages and T cells (7, 19, 20, 34). RANTES could also be implicated in type 2 diabetes development (15). We have shown previously that the megakaryocytic growth factor TPO was oversecreted by omental adipose tissue of obese subjects (25) and that its circulating levels were increased in obesity and negatively correlated with adiponectinemia (24). Herein, we extend those data by demonstrating that TPO is unambiguously regulated in adipocytes by ApN excess or deficiency in vivo. Because high systemic TPO levels could participate in the pathogenesis of the acute coronary syndrome (33), it is tempting to speculate that suppressed TPO may further link ApN to cardiovascular protection. Like TPO, ApN specifically downregulated VEGFR1. This downregulation is of interest since inactivation of a VEGF homolog, which specifically binds VEGFR1, led to impaired AT development via reduced angiogenesis in obese rodents (21). The only adipokine that was specifically upregulated by ApN in the present study was IL-1R4, a member of the Toll-like 1 receptor family. However, we cannot exclude the possibility that some other anti-inflammatory adipokines, like IL-10, whose levels were very low by cytokine antibody arrays (our own data) may have escaped the detection. Herein, both soluble secreted form and total

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**Fig. 5.** Gene expression of adipokines in adipocytes (A) and SVC (B) from ApN-Overex mice. mRNA levels of the adipokines, which were measured by ELISA in Fig. 4, were quantified by RT-qPCR. Values were normalized to the levels of cyclophilin and presented as relative expression compared with WT mice. Results are presented as means ± SE for 6–7 mice/group. *P < 0.05 vs. WT mice.
gene expression of IL-1R4 were enhanced by ApN. IL-1R4 may act as an anti-inflammatory factor; it suppressed inflammatory responses induced by LPS both in vitro and in vivo (36), whereas IL-1R4-deficient mice produced elevated concentrations of pro-inflammatory cytokines and failed to develop endotoxin tolerance (5). Recently, a protective role for IL-1R4 has been proposed in atherosclerosis (28) and obesity as well (27). IL-1R4 protected obese mice against the development of AT inflammation and related metabolic disorders (27). Thus, several adipokines identified as downstream targets of ApN may be instrumental in further deciphering the pathogenesis of the metabolic syndrome.

After identifying these new secretory products, we examined whether their regulation occurred at the pretranslational level. We thus measured the mRNA levels of all of these adipokines. Gene expression pattern of most adipokines roughly followed the secretion pattern, indicating a pretranslational effect of ApN. Expression of some genes, like TPO, IL-12p70, VEGFR1, ICAM-1, and IL-1R4, further showed a reverse pattern in the ApN-lacking condition, implying that ApN was the specific regulator of these changes. However, the expression of some genes was not affected in ApN-deficient mice.

Yet the two models of mice were not the exact opposite of each other. In ApN-KO mice there is a complete and generalized lack of ApN, and some compensatory mechanisms may operate and mask the expected repercussions, especially on AT [e.g., these mice do not develop obesity (22)]. In ApN-Overex mice, the overexpression is targeted primarily to white AT and remains moderate (i.e., within a physiological range).

To unravel the mechanisms underlying the anti-inflammatory effects of ApN on AT, we explored several potential intracellular signaling pathways linking inflammation/immune response to obesity. Obesity and high-fat diet are known to activate IKKβ/NF-κB, JNK, and ERK1/2 pathways in AT (17, 35). On the other hand, AMPK activation is a crucial link in the signaling effects of ApN in metabolically responsive tissues, including AT (41); thus, we also explored the AMPK pathway. ApN has been reported to inhibit LPS-stimulated activation of NF-κB, JNK, and ERK1/2 (or related MAP kinase) in cultured porcine or human blood-derived macrophages (10, 42). We confirmed in vivo that ApN indeed suppressed the activation of NF-κB and ERK1/2 in white AT, although it did not affect JNK protein or phosphorylation. We also found that
ApN enhanced both AMPK protein expression and phosphorylation. Most studies focused on ApN-induced AMPK phosphorylation and hardly characterized the relationship between ApN and AMPK protein expression. The increased AMPK protein expression found herein is likely to result from chronic exposure to ApN. Noticeably, AMPK activity has recently been found to be reduced in omental AT of obese subjects and to be associated with markers of inflammation (12). Hence, the decreased activity of NF-κB and ERK1/2 as well the increased expression and activity of AMPK were associated with, and were likely to contribute to, the protective effect of ApN against inflammation into AT.

In conclusion, ApN regulates in vivo the secretion of several newly identified downstream adipokines, thereby inducing a shift of the immune balance in both adipocytes and SVC toward a less inflammatory phenotype. These downstream adipokines may be new therapeutic targets for the management of the metabolic syndrome.

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DISCLOSURES

The authors have nothing to disclose.

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