Interactions Between Vascular Wall and Perivascular Adipose Tissue Reveal Novel Roles for Adiponectin in the Regulation of Endothelial Nitric Oxide Synthase Function in Human Vessels

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Background—Adiponectin is an adipokine with potentially important roles in human cardiovascular disease states. We studied the role of adiponectin in the cross-talk between adipose tissue and vascular redox state in patients with atherosclerosis.

Methods and Results—The study included 677 patients undergoing coronary artery bypass graft surgery. Endothelial function was evaluated by flow-mediated dilation of the brachial artery in vivo and by vasomotor studies in saphenous vein segments ex vivo. Vascular superoxide (O$_2^-$) and endothelial nitric oxide synthase (eNOS) uncoupling were quantified in saphenous vein and internal mammary artery segments. Local adiponectin gene expression and ex vivo release were quantified in perivascular (saphenous vein and internal mammary artery) subcutaneous and mesothoracic adipose tissue from 248 patients. Circulating adiponectin was independently associated with nitric oxide bioavailability and O$_2^-$ production/eNOS uncoupling in both arteries and veins. These findings were supported by a similar association between functional polymorphisms in the adiponectin gene and vascular redox state. In contrast, local adiponectin gene expression/release in perivascular adipose tissue was positively correlated with O$_2^-$ and eNOS uncoupling in the underlying vessels. In ex vivo experiments with human saphenous veins and internal mammary arteries, adiponectin induced Akt-mediated eNOS phosphorylation and increased tetrahydrobiopterin bioavailability, improving eNOS coupling. In ex vivo experiments with human saphenous veins/internal mammary arteries and adipose tissue, we demonstrated that peroxidation products produced in the vascular wall (ie, 4-hydroxynonenal) upregulate adiponectin gene expression in perivascular adipose tissue via a peroxisome proliferator-activated receptor-γ-dependent mechanism.

Conclusions—We demonstrate for the first time that adiponectin improves the redox state in human vessels by restoring eNOS coupling, and we identify a novel role of vascular oxidative stress in the regulation of adiponectin expression in human perivascular adipose tissue. (Circulation. 2013;127:2209-2221.)

Key Words: 5,6,7,8-tetrahydrobiopterin ▪ adiponectin ▪ atherosclerosis ▪ endothelium ▪ nitric oxide synthase type III ▪ superoxides

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Experimental studies suggest that adiponectin stimulates nitric oxide (NO) production in endothelial cell cultures. This is believed to be due in part to endothelial NO synthase (eNOS) activation through PI3 kinase/Akt-mediated phosphorylation. However, whether adiponectin exerts the same effects in the human vasculature remains to be established. Indeed, the biological role of adiponectin appears to be much more complex in human cardiovascular disease than in experimental models. In particular, the possible protective effect of high circulating adiponectin in healthy individuals is lost (or even reversed) in advanced cardiovascular disease states such as heart failure.

Under conditions of increased vascular oxidative stress observed in human atherosclerosis, eNOS is uncoupled mostly as a result of oxidation of its cofactor tetrahydrobiopterin (BH4) and produces superoxide radicals (O₂⁻) instead of NO. In this biological setting, activation of eNOS by adiponectin may increase O₂⁻ generation from uncoupled eNOS if there is no parallel increase in vascular BH4. Therefore, the biological role of adiponectin in the regulation of eNOS functional status and activity in human atherosclerosis remains unexplored.

In this study, we examine the impact of adiponectin and vascular NO bioavailability/redox state in patients with advanced atherosclerosis and investigate, for the first time in humans, the relationship between local adiponectin synthesis in perivascular adipose tissue (AT) and O₂⁻ generation in patients with coronary artery bypass graft surgery, and the vasorelaxations in response to acetylcholine and sodium nitroprusside were studied in an organ-bath setting as described previously (see Methods in the online-only Data Supplement).

**Methods**

**Population and Protocol of Study 1**

The population of study 1 consisted of 677 patients (Table) undergoing elective coronary artery bypass graft surgery. The day before surgery, endothelium-dependent flow-mediated dilation (FMD) and endothelium-independent dilatation of the brachial artery were determined (see Methods in the online-only Data Supplement). Fasted blood samples were obtained on the morning of the surgery. During coronary artery bypass graft surgery, segments of saphenous vein (SV) and internal mammary artery (IMA) were obtained. Exclusion criteria were any inflammatory, infectious, liver, or renal disease or malignancy. Patients with recent unstable coronary syndrome (within the previous 8 weeks) or clinical heart failure syndrome and those receiving nonsteroidal anti-inflammatory drugs, dietary supplements, or antioxidant vitamins were also excluded.

In a subgroup of 248 patients, samples of perivascular AT surrounding the IMA (peri–IMA-AT) and SV (peri–SV-AT), subcutaneous AT (Sc-AT; from the site of the chest incision), and mesoatocathoracic AT (Ms-AT; attached to the pericardium) were also obtained. AT samples from all sites were snap-frozen and stored at −80°C for gene expression studies or were used for tissue culture experiments (Sc-AT, peri–SV-AT, and Ms-AT) as described below.

The study was approved by the Institutional Review Committee. All subjects gave written informed consent.

**Blood Sampling and Adiponectin Measurements**

Venous blood samples were obtained after 8 hours of fasting on the morning of the surgery, and serum adiponectin was measured by ELISA (see Methods in the online-only Data Supplement).

**DNA Extraction and Genotyping**

Genomic DNA extraction from whole blood and genotyping were performed by standard methodology (see Methods in the online-only Data Supplement).

**Vessel Harvesting and Vasomotor Studies**

Human SV and IMA samples were obtained at the time of coronary artery bypass graft surgery, and the vasorelaxations in response to acetylcholine and sodium nitroprusside were studied in an organ-bath setting as described previously (see Methods in the online-only Data Supplement).

**Vascular Super oxide Measurements**

Vascular O₂⁻ production was measured in fresh, intact IMA and SV segments by using lucigenin (5 µmol/L)-enhanced chemiluminescence, as previously described (see Methods in the online-only Data Supplement).

**Adipose Tissue Culture**

Samples of Sc-AT, Ms-AT, and peri–SV-AT obtained from patients in study 1 were used to estimate the biosynthetic rate of adiponectin in an ex vivo bioassay (see Methods in the online-only Data Supplement). AT from these depots was routinely cultured for 4 hours, and culture supernatants were collected to estimate the release of adiponectin.

**RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction**

Samples of Sc-AT, Ms-AT, peri–SV-AT, and peri–IMA-AT were used for gene expression studies (see Methods in the online-only Data Supplement).

**Measurement of Vascular Biopterins**

Vascular BH4, dihydrobiopterin, and biotin levels were each determined separately from the same sample through the use of high-performance liquid chromatography followed by serial electrochemical and fluorescent detection, as we have previously described (see Methods in the online-only Data Supplement).

**Population in Study 2 and Experimental Procedures**

To examine the direct effects of adiponectin on the mechanisms regulating NO bioavailability and O₂⁻ production in human vessels, we used ex vivo models of human SVs and IMAs, as previously described. For these experiments we recruited 46 patients undergoing coronary artery bypass graft surgery (Table) using the same exclusion criteria as for study 1. Serial SV/IMA segments were incubated ex vivo in the presence or absence of recombinant full-length adiponectin 10 µg/mL for 6 hours. The effect of adiponectin on vascular O₂⁻ (basal and N-nitro-l-arginine methyl ester [LNAME]–inhibitable O₂⁻) was quantified by lucigenin-enhanced chemiluminescence and visualized with oxidative fluorescent dye, dihydroethidium, staining (see below). The changes in Akt and eNOS phosphorylation status were determined by Western blotting; vascular biopterins were quantified by high-performance liquid chromatography, as described above. In additional studies, SV and IMA segments were incubated with adiponectin 10 µg/mL in the presence and absence of wortmannin (100 nmol/L) to inhibit PI3 kinase/Akt signaling or 2,4-diaminohydroxyl pyrimidine (DAHP) to inhibit GTP-cyclodihydrolase (the rate-limiting enzyme in the biosynthetic pathway of biopterins; see Methods in the online-only Data Supplement).

To examine the impact of vascular oxidative stress on local adiponectin synthesis in perivascular AT, we first incubated peri–SV-AT and peri–IMA-AT with 4-hydroxynonenal (4-HNE; 30 µmol/L) for 16 hours in the presence and absence of the PPAR-γ inhibitor T0070907 (10 µmol/L for peri–SV-AT) and examined the changes in ADIPOQ, PPAR-γ, and CD36 (which is downstream of PPAR-γ) gene expression. To examine whether 4-HNE is produced by human
Table. Demographic Characteristics of Study Participants

<table>
<thead>
<tr>
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<th>Clinical Studies (Study 1)</th>
<th>Ex Vivo Studies (Study 2)</th>
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<tr>
<td>Participants, n</td>
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<tr>
<td>Age, y</td>
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<td>Male sex, n (%)</td>
<td>563 (83)</td>
<td>359 (78)</td>
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<td>Hypertension, n (%)</td>
<td>465 (68)</td>
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<td>Hyperlipidemia, n (%)</td>
<td>433 (64)</td>
<td>22 (49)</td>
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<td>Diabetes mellitus, n (%)</td>
<td>221 (33)</td>
<td>12 (27)</td>
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<tr>
<td>Smoking (active/ex), n (%)</td>
<td>161/325 (24/48)</td>
<td>8/20 (18/44)</td>
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<tr>
<td>BMI, kg/m²</td>
<td>27.6±0.17</td>
<td>29.3±0.72</td>
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<td>Cholesterol, mg/dL</td>
<td>177.7±2.0</td>
<td>155.8±16.3</td>
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<td>HDL, mg/dL</td>
<td>38.6±0.5</td>
<td>36.3±3.9</td>
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<td>Triglycerides, mg/dL*</td>
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<td>107 (56–126)</td>
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<tr>
<td>Medication, %</td>
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<td>ACEI/ARBs</td>
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<tr>
<td>CCBs</td>
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</table>

ACEI indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blockers; BMI, body mass index; CCB, calcium channel blockers; and HDL, high-density lipoprotein. Values are expressed as mean±SEM.

SVs and IMAs in the presence of oxidative stress, we quantified 4-HNE protein adducts content in 11 SVs and 18 IMAs and related these measurements to vascular O$_2^-$ production from these vessels (see Methods in the online-only Data Supplement).

Oxidative Fluorescent Microtopography

In situ O$_2^-$ production was determined in vessel cryosections by oxidative fluorescent dye, dihydroethidium, staining, as previously described (see Methods in the online-only Data Supplement).

Western Blots

Western blots in human vessels for Akt/phosphorylated Akt (Ser473), eNOS/phosphorylated eNOS (Ser1177), and 4-HNE adducts were performed as described in Methods in the online-only Data Supplement.

Statistical Analysis

Continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test. Nonnormally distributed variables were log-transformed for analysis.

Sample size calculations were based on previous data from our laboratory. For the clinical studies, we estimated that a total number of 500 subjects would allow us to detect a 2.38 absolute difference in FMD between the highest and lowest tertiles of circulating adiponectin with an α =0.05, a power of 0.9, and an assumed standard deviation of 2.5. For the AT experiments, we estimated that with 150 patients we would be able to detect a 0.13 difference in log(O$_2^-$) in SV samples between the extreme tertiles of adiponectin released from peri–SV-AT with an α =0.05, a power of 0.9, and an assumed standard deviation of 0.2. For the ex vivo experiments, sample size calculations were performed on the basis of our previous experience on this model, and we estimated that with a minimum of 5 pairs of samples (serial rings from the same vessel) we would be able to identify a change in log(O$_2^-$) of 0.48 with an α =0.05, a power of 0.9, and a standard deviation for a difference in the response of the pairs of 0.25.

In the clinical studies, continuous variables between 3 groups were compared by use of 1-way ANOVA followed by the Bonferroni post hoc test for individual comparisons, whereas comparisons between 2 groups were performed by unpaired t tests. Categorical variables were compared by use of the χ² test as appropriate. Correlations between continuous variables were assessed by bivariate analysis, and the Pearson coefficient was estimated. For the organ-bath experiments, the effect of circulating adiponectin tertile on vasorelaxations in response to acetylcholine was evaluated by use of 2-way ANOVA for repeated measures (examining the effect of acetylcholine or sodium nitroprusside concentration by circulating adiponectin tertile or genotype interaction on vasorelaxations) in a full factorial model. For the ex vivo experiments (in which serial rings from the same vessel were incubated with multiple interventions, we performed repeated measures ANOVA and paired t tests for individual comparisons, followed by the Bonferroni post hoc correction for multiple testing as appropriate.

In the clinical studies, correlations between continuous variables were tested by calculating the Pearson correlation coefficient. Linear regression was performed by using as dependent variables FMD or log(vascular O$_2^-$) and as independent variables log(circulating adiponectin) and the clinical demographic characteristics (age, sex, diabetes mellitus, smoking, dyslipidemia, hypertension) that showed a simple association with the dependent variable at the level of 15%. A backward elimination procedure was then used by having P =0.1 as the threshold to remove a variable from the respective model. All statistical tests were performed with SPSS version 20.0, and values of P <0.05 were considered statistically significant.

Results

Adiponectin and NO-Mediated Vasorelaxations in Human Vessels

We first examined the association between circulating adiponectin and endothelial function (as evaluated by FMD and vasorelaxations of SV rings ex vivo). Circulating adiponectin was positively correlated with FMD (Figure 1). In multivariable analysis, the independent predictors of FMD were log(circulating adiponectin) (β, 1.75; SE, 0.69; P =0.012), hypertension (β, −2.16; SE, 0.47; P =0.0001), and smoking (β, −1.51; SE, 0.31; P =0.0001). This finding was confirmed in organ-bath studies in which the vasorelaxations of SV segments in response to acetylcholine were significantly greater in vessels from patients in the highest tertile of circulating adiponectin compared with patients in the lowest tertile. There were no associations between circulating adiponectin and either endothelium-independent dilatation of the brachial artery in vivo or vasorelaxations of SVs in response to sodium nitroprusside ex vivo (Figure 1).

Circulating Versus Local Adiponectin in Perivascular AT and Vascular Superoxide

We next examined whether circulating adiponectin was related to vascular O$_2^-$ generation in human arteries (IMA) and veins (SV). We observed that circulating adiponectin was closely related to basal O$_2^-$ in both vessel types (Figure 2). In multivariable analysis, the independent predictors of log(O$_2^-$) in SV were log(circulating adiponectin) (β, −0.2984; SE, 0.06; P =0.0001), smoking (β, 0.068; SE, 0.024; P =0.005), and treatment with statins (β, −0.219; SE, 0.044; P =0.0001). Similarly, the independent predictors of log(O$_2^-$) in IMAs were log(circulating adiponectin) (β, −0.265; SE, 0.077; P =0.001), statin treatment (β, −0.199; SE, 0.061; P =0.001), diabetes mellitus (β, 0.112; SE, 0.047; P =0.019), and smoking (β, 0.053; SE, 0.032; P =0.097).
To test for possible paracrine effects of local adiponectin secreted by perivascular AT, we quantified both the expression of ADIPOQ gene and local adiponectin protein secretion by peri-SV-AT after 4 hours of culture ex vivo and tested for their associations with vascular redox state. In contrast to what was observed with circulating adiponectin, increased vascular O$_2^-$ in human SVs and IMAs was related to increased expression of the ADIPOQ gene and local adiponectin secretion from the perivascular AT (Figure 2). These discordant relationships between vascular O$_2^-$ production and circulating versus local adiponectin production in perivascular AT (confirmed in both human arteries and veins) suggest that local production of adiponectin in perivascular AT and circulating adiponectin are differentially regulated and imply that vascular O$_2^-$ has the potential to stimulate local adiponectin production in the neighboring perivascular AT.

Circulating Versus Local Adiponectin in Perivascular AT and eNOS Coupling
To assess whether there are interactions between circulating/local adiponectin and eNOS coupling, we examined the association between both circulating adiponectin and its local expression (in peri-SV-AT and peri-IMA-AT) and LNAME-inhibitable vascular O$_2^-$ (which provides an estimate of eNOS uncoupling). We observed that low circulating adiponectin levels were related to greater LNAME-inhibitable O$_2^-$ in both SV and IMA segments, which is indicative of more eNOS uncoupling (Figure 3). In multivariable analysis, we observed that the independent predictors of LNAME-Δ(O$_2^-$) in IMAs were log(circulating adiponectin) (β, 4.37; SE, 0.73; P=0.0001) and treatment with statins (β, 2.73; SE, 0.57; P=0.0001), whereas the predictors of LNAME-Δ(O$_2^-$) in SVs were also log(circulating adiponectin) (β, 2.12; SE, 0.54; P=0.0001) and statin treatment (β, 2.25; SE, 0.39; P=0.0001). These findings suggest that increased circulating adiponectin and regular statin treatment are independently associated with improved eNOS coupling in these human arteries and veins. In contrast, we observed that higher LNAME-inhibitable O$_2^-$ in either SVs or IMAs (indicative of more eNOS uncoupling) was related to higher ADIPOQ gene expression in the perivascular AT (peri-SV-AT and peri-IMA-AT) of these vessels, a finding compatible with the increased local adiponectin synthesis observed in peri-SV-AT from these patients (Figure 3).

Linking Circulating and Local Adiponectin Production in AT: The Role of PPAR-γ
To further explore the possible contribution of the different AT depots to the circulating adiponectin pool, we examined the association between circulating adiponectin and its local release/ADIPOQ gene expression in Ms-AT, Sc-AT, peri-SV-AT, and peri-IMA-AT. We observed a significant, albeit weak, correlation between local adiponectin release and ADIPOQ gene expression in Ms-AT and Ac-AT, suggesting that the circulating pool may be driven partly by adiponectin produced by these remote (nonperivascular) depots (Figure 4). In contrast, circulating adiponectin was not related to either local adiponectin release/ADIPOQ gene expression in peri-SV-AT (Figure 4) or ADIPOQ gene expression in peri-IMA-AT (r=0.072, P=0.602), implying that different,
probably local, mechanisms control the release of adiponectin in perivascular AT.

To further explore the subcellular mechanisms controlling ADIPOQ gene expression in the various AT depots, we quantified the expression of PPAR-\(\gamma\), which is known to regulate ADIPOQ gene expression in cell culture models.\(^{18}\) We observed a strong correlation between PPAR-\(\gamma\) and ADIPOQ gene expression in peri–SV-AT (\(r=0.576, P<0.0001\)), peri–IMA-AT (\(r=0.751, P<0.0001\)), Ms-AT (\(r=0.514, P<0.0001\)), and Sc-AT (\(r=0.344, P<0.0001\)). To confirm that PPAR-\(\gamma\) gene expression provides a good estimate of PPAR-\(\gamma\) activity,\(^{19}\) we then quantified the expression of its downstream molecule, CD36 (known to be highly regulated by PPAR-\(\gamma\) activity),\(^{19}\) and confirmed collinearity between the expression of PPAR-\(\gamma\) and CD36 genes in peri–SV-AT (\(r=0.976, P<0.0001\)), peri–IMA-AT (\(r=0.956, P<0.0001\)), Ms-AT (\(r=0.933, P<0.0001\)), and Sc-AT (\(r=0.953, P<0.0001\)). These results confirm that ADIPOQ gene expression remains under the direct control of PPAR-\(\gamma\) in all types of human AT studied.

**Effects of Adiponectin on Endothelial Function and Vascular Redox State by Using the Genetic Variability of ADIPOQ**

To explore the discordant associations between vascular \(O_2^-\) and circulating levels and local (perivascular) adiponectin biosynthesis, we searched for genetic single nucleotide polymorphisms with a known impact on adiponectin circulating levels. The genetically determined variability of adiponectin levels could then be used as a model system to test indirectly the effect of adiponectin on vascular NO bioavailability and vascular \(O_2^-\) generation in human vessels. We genotyped the entire population in study 1 for 2 common genetic polymorphisms: rs17366568 in ADIPOQ and rs266717 in ADIPOQ gene promoter, both known to affect circulating adiponectin levels in genome-wide association studies.\(^{20,21}\) These 2 polymorphisms were not in linkage disequilibrium (data not shown), so their effect on ADIPOQ gene expression could be independent from each other and therefore additive. Indeed, we observed that the number of rs266717T plus rs17366568G alleles was positively associated with FMD, the ex vivo vasorelaxations in response to acetylcholine, and total/LNAME-inhibitable \(O_2^-\) in SV and IMA segments (indicative of eNOS uncoupling when the genetic background leads to lower circulating adiponectin). These findings document for the first time that genetically determined hypoadiponectinemia may actually lead to endothelial dysfunction and eNOS uncoupling in human vessels.
Direct Effects of Adiponectin on Redox State and eNOS Coupling by Using Ex Vivo Models of Human Vessels

To further explore the discordant associations between vascular endothelial function/redox state and circulating/local adiponectin production in perivascular AT (in study 1), we performed a number of mechanistic experiments in human IMAs and SVs ex vivo (study 2). We first incubated segments of these vessels with or without adiponectin 10 μg/mL for 6 hours and examined its effect on basal and LNAME-inhibitable O2−. Adiponectin induced a striking reduction in basal O2− by restoring eNOS coupling in both human SVs and IMAs (Figure 6). This effect was also confirmed by dihydroethidium staining of these vessels, in which adiponectin rapidly reversed LNAME-inhibitable O2− in the endothelium (Figure 6).

To further explore the mechanisms by which adiponectin affects eNOS physiology in the human endothelium, we explored the impact on eNOS phosphorylation in these vessels. Ex vivo incubation of human SVs and IMAs with adiponectin increased Akt phosphorylation at Ser473 and eNOS phosphorylation at Ser1177 (Figure 7). The effect of adiponectin on eNOS phosphorylation was blocked by wortmannin, an inhibitor of PI3-mediated Akt phosphorylation, suggesting that adiponectin induces a PI3-Akt-mediated phosphorylation of eNOS, resulting in its activation. The ability of adiponectin to induce eNOS phosphorylation remained unchanged in the presence of DAHP (an inhibitor of GTP-cyclohydrolase blocking the biosynthetic pathway of biopterins), suggesting that a reduction in vascular BH4 levels does not modify the effect of adiponectin on eNOS phosphorylation.

However, activation of eNOS by Ser1177 phosphorylation does not necessarily increase NO synthesis because phosphorylated eNOS in the presence of BH4 deficiency may even lead to increased O2− generation by the uncoupled enzyme.15 Therefore, we examined whether circulating adiponectin was related to vascular BH4 content. In a subgroup of 176 patients from study 1, circulating adiponectin was positively correlated with BH4 and the ratio of BH4 to total biopterins in both SV and IMA segments (Figure 7). Previous reports suggest that BH4 administration increases circulating adiponectin in an animal model,22 but it is unclear whether circulating adiponectin has any direct effect on vascular BH4 bioavailability in humans. We observed a significant but weak association between circulating adiponectin and vascular biopterins in study 1 (Figure 7). To further explore this association, we incubated human SVs and IMAs with adiponectin (10 μg/mL) for 6 hours. Adiponectin increased vascular BH4 and the ratio of BH4 to total biopterins in human SVs (Figure 7), whereas inhibition of GTP-cyclohydrolase with DAHP resulted in the expected reduction of vascular BH4 levels, even in the presence of adiponectin. Taken together, these findings suggest that adiponectin improves NO and reduces O2− bioavailability in human vessels through a combined effect on eNOS activation (via PI3/Akt phosphorylation) and coupling (by increasing BH4 bioavailability).
Effects of Vascular Oxidative Stress on Adiponectin Expression in Perivascular AT

To explore the positive association observed between $\text{O}_2^-$ production in human SVs/IMAs and adiponectin release/ADIPOQ expression in the respective perivascular AT, we then examined whether products of peroxidation released from the vascular wall may regulate ADIPOQ expression in perivascular AT. Recent data suggested that 4-HNE (a product of lipid peroxidation) may upregulate ADIPOQ gene expression in both primary human adipocytes and skeletal muscle cells; therefore, we hypothesized that 4-HNE may be involved in the cross-talk between vascular wall and perivascular AT in humans. To examine whether increased vascular $\text{O}_2^-$ leads to increased 4-HNE production in human vessels, we first quantified the levels of 4-HNE protein adducts in SV (n=11) and IMA (n=18) segments from our cohort in study 1 and demonstrated a good correlation between vascular $\text{O}_2^-$ and 4-HNE protein adducts in both SVs ($r=0.720, P=0.042$) and IMAs ($r=0.489, P=0.039$; representative examples are shown in Figure 8), suggesting that there is increased production of 4-HNE in human arteries and veins in the presence of increased vascular oxidative stress. We then incubated peri–SV-AT (n=6) and peri–IMA-AT (n=6) with 4-HNE 30 µmol/L for 16 hours and observed that ADIPOQ gene expression was upregulated by $\approx 2$ fold in both peri–SV-AT (Figure 8) and peri–IMA-AT (by $1.9\pm0.3$ fold; $P<0.05$ versus control). This was accompanied by a parallel upregulation of PPAR-$\gamma$ activity at 10 µmol/L, the effect of 4-HNE on ADIPOQ gene expression in peri–SV-AT was abolished (Figure 8).

As a positive control in these experiments, we used CD36 (a downstream molecule with an expression that is under the direct control of PPAR-$\gamma$), which showed responses similar to ADIPOQ in both peri–SV-AT (Figure 8) and peri–IMA-AT (upregulated by $1.8\pm0.4$ fold after incubation with 4-HNE). These experiments suggest that 4-HNE produced in human SVs and IMAs in the presence of increased vascular oxidative stress may upregulate the expression of ADIPOQ gene in perivascular AT through a PPAR-$\gamma$–dependent mechanism.

Discussion

In the present study, we examine the role of both circulating and locally produced adiponectin in the regulation of vascular redox state in patients with atherosclerosis. We demonstrate for the first time in humans that, in addition to its association...
with NO bioavailability, circulating adiponectin is inversely related to vascular $\text{O}_2^-$ (derived from uncoupled eNOS) in human arteries and veins, independently of atherosclerosis risk factors. These findings are also confirmed by linking the genetic variability of $\text{ADIPOQ}$ with vascular redox state and NO bioavailability and by using ex vivo experiments with human arteries and veins. We further demonstrate that the effect of adiponectin on eNOS coupling is mediated by its combined impact on PI3/Akt-mediated phosphorylation of eNOS and vascular BH4 bioavailability. In contrast, increased vascular $\text{O}_2^-$ is associated with increased local adiponectin release/$\text{ADIPOQ}$ gene expression in perivascular AT, implying that local mechanisms related to the vascular redox state may control adiponectin synthesis in perivascular AT in patients with atherosclerosis. In additional ex vivo experiments in human vessels and perivascular AT, we demonstrated that vascular oxidative stress induces the release of products of lipid peroxidation (ie, 4-HNE) that upregulate $\text{ADIPOQ}$ gene expression.

Figure 5. The number of G alleles for rs17366568G (polymorphism in $\text{ADIPOQ}$ gene) and T alleles for rs266717 (polymorphism in $\text{ADIPOQ}$ promoter region) had an additive effect on circulating adiponectin (AdN) levels (n=677; A). A similar effect was also observed for AdN release by mesothoracic (Ms-AT; B) but not perivascular (peri–SV-AT, C) adipose tissue (AT). The number of rs17366568G and rs266717T alleles was also positively associated with flow-mediated dilation (FMD) of the brachial artery (D) and vasorelaxations of saphenous veins (SVs; n=230) in response to acetylcholine (ACh; E) but not to sodium nitroprusside (SNP; F). The number of rs17366568G and rs266717T alleles was also related to less vascular superoxide ($\text{O}_2^-$; G) and less N-nitro-L-arginine methyl ester (LNAME)–inhibitable $\text{O}_2^-$ (H) in both SVs (n=396) and internal mammary arteries (IMAs; n=257). Values are expressed as median (25th–75th percentiles) in A through C and G and as mean±SEM in D through F and H.
in the perivascular AT via a PPAR-γ–dependent mechanism. This cycle of cross-talk between the vessel and perivascular AT may represent a novel defense mechanism of the human vascular wall against oxidative stress (Figure 8).

Adiponectin and Endothelial Dysfunction
Adiponectin is an adipokine with antiatherogenic properties in experimental models but has a controversial role in the clinical setting. Although circulating adiponectin is reduced in obesity and is related to reduced cardiovascular risk in the general population, increased circulating adiponectin is inversely correlated with mortality and the overall clinical outcome in advanced cardiovascular disease states such as heart failure. Moreover, pharmacological treatments that improve endothelial function and reduce cardiovascular risk (such as lipophilic statins) appear to reduce circulating adiponectin in patients with hypercholesterolemia. This discordance between experimental data and clinical observations has introduced the concept that adiponectin may behave as a rescue hormone in advanced disease states, although the mechanisms behind this hypothesis have not been explored in the clinical setting.

Experimental evidence suggests that adiponectin stimulates eNOS-derived NO in endothelial cells through stimulation of PI3/Akt-mediated eNOS phosphorylation. In keeping with these first observations from cell culture models, adiponectin knockout mice exhibit reduced eNOS phosphorylation status (at Ser1177) and impaired endothelial function. However, the relevance of these observations to humans is totally unknown.

In clinical studies, circulating adiponectin is directly related to endothelial function in the general population, but this association is reversed in patients with type 2 diabetes mellitus and diabetic nephropathy, introducing further controversy into the role of adiponectin in different clinical disease states.

In the present study, we demonstrate that circulating adiponectin is an independent predictor of endothelial function in a well-phenotyped cohort of patients with coronary artery disease without renal or heart failure. In the first attempt to prove a causal link between adiponectin and endothelial function, we examined whether genetic polymorphisms known from genome-wide association studies to regulate circulating adiponectin levels (rs17366568 in ADIPOQ and rs266717 in ADIPOQ promoter) had an impact on endothelial function.

We observed that genetically determined lower circulating adiponectin levels were related to worse endothelial function. To further investigate the molecular mechanisms underlying this observation, we exposed human SVs and IMAs to adiponectin ex vivo and observed a rapid PI3/Akt-mediated increase in eNOS phosphorylation at Ser1177, which is known to activate the enzyme. Although eNOS is the source of NO in the vascular endothelium, deficiency of its cofactor BH4 leads to its uncoupling, turning it into a source of $O_2^-$ instead of NO. We demonstrate for the first time that adiponectin increases vascular BH4, a critical cofactor necessary for eNOS enzymatic coupling in human vessels. Although the biological significance of circulating adiponectin in the regulation of vascular BH4 bioavailability in humans in
vivo needs further validation, our finding is compatible with the strong association we observe between circulating adiponectin and eNOS coupling in human arteries and veins. Therefore, it is likely that even this rather weak effect of adiponectin on vascular biopterins may have a significant impact on eNOS coupling in vivo. The combined phosphorylation of eNOS and increase in its enzymatic coupling also explain the impact of circulating adiponectin on vascular NO bioavailability in humans.

**Adiponectin and Vascular Superoxide Generation**

Evidence from cell culture models suggests that activation of eNOS via Ser1177 phosphorylation leads to either increased $O_2^-$ or NO, depending on the underlying coupling status of the...
enzyme.15 Given that vascular BH4 is oxidized by free radicals and the balance between its synthesis and oxidation defines its net bioavailability,12 any change in vascular redox state could drive eNOS uncoupling and trigger a proatherogenic vicious cycle.

We demonstrate for the first time in humans that circulating adiponectin is an independent predictor of vascular $O_2^-$ and eNOS coupling in arteries and veins from patients with coronary atherosclerosis. By using the genetic variability of ADIPOQ and ex vivo models of human arteries and veins, we also demonstrate that adiponectin reduces vascular $O_2^-$ by its combined impact on BH4-mediated eNOS coupling and PI3/Akt-mediated activation of the enzyme.

**Effects of Vascular Oxidative Stress on Adiponectin Expression in Perivascular AT**

Evidence suggests that ischemia/reperfusion injury leads to increased adiponectin expression in brain vessels,31 a vascular bed where uncoupled eNOS is a key contributor to vascular $O_2^-$ generation.32 Moreover, it has recently been shown that 4-HNE, a product of peroxidation released from tissues with high oxidative burden, upregulates ADIPOQ in primary human and 3T3 adipocytes,33 as well as in skeletal muscle.23 These findings support the notion that adiponectin may behave as a rescue hormone, being upregulated in conditions of increased oxidative stress.

In our study, vascular $O_2^-$ production (in both arteries and veins) was paradoxically positively correlated with adiponectin release and ADIPOQ gene expression in perivascular AT. We have shown that peroxidation products (ie, 4-HNE, produced in the vascular wall in the presence of increased vascular oxidative stress) upregulate ADIPOQ gene in perivascular AT in a PPAR-γ-dependent mechanism. These findings introduce the concept of a novel, local defense mechanism of the vascular wall against oxidative stress that relies on the continuous cross-talk between the vessel and its perivascular fat.

Given that both vessel types used in our study (IMA and SV) were free of atherosclerosis, it is unclear whether these findings are applicable to the human coronary arteries, especially in the presence of coronary atherosclerosis. Although our ex vivo models use human vessels obtained from patients with advanced atherosclerosis (therefore chronically exposed...
to a proatherogenic environment), the absence of atherosclerotic lesions in these vessels is a potential limitation of the study. Moreover, the extent to which adiponectin produced in peripheral AT is sufficient to effectively control redox state in the human vascular wall in vivo is hard to estimate, given the complexity of the mechanisms implicated in redox state regulation in the human vascular wall. Finally, it is unclear whether our findings are applicable to human arteries and veins from healthy individuals because these vessels are harvested only during coronary artery bypass graft surgery, and these patients are also under multiple pharmacological treatments.

Conclusions
This is the first study demonstrating that adiponectin has a direct impact on redox state in human arteries and veins through its combined effect on BH4-mediated improvement of eNOS coupling and PI3/Akt-mediated phosphorylation of eNOS. It also introduces the novel concept that increased oxidative stress in the vessel wall leads to the release of peroxynitrite. It upregulates adiponectin gene expression in perivascular AT via a PPARγ-dependent mechanism. These findings propose for the first time a bidirectional cross-talk between the human vascular wall and perivascular AT, with potentially important implications in vascular biology.

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Disclosures
None.

References

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**CLINICAL PERSPECTIVE**

Obesity is a risk factor for atherosclerosis, but the molecular mechanisms by which the various depots of adipose tissue contribute to atherogenesis are unclear. Adiponectin, an adipokine with a circulating level that is reduced in obesity, appears to have potentially important roles in human cardiovascular disease states. In this study, we explored the role of adiponectin in the cross-talk between adipose tissue and vascular redox state in patients with atherosclerosis. In a large clinical cohort undergoing coronary artery bypass graft surgery and ex vivo models of human arteries and veins obtained during coronary artery bypass graft surgery, we demonstrate a novel role of adiponectin as a key regulator of redox state in human vessels. Adiponectin stimulates the activity and improves enzymatic coupling of endothelial nitric oxide synthase in the human vascular endothelium, reducing vascular superoxide generation while increasing nitric oxide bioavailability. We also demonstrate that peroxidation products from the human vascular wall (ie, 4-hydroxynonenal) upregulate the expression of adiponectin in the local perivascular adipose tissue, which may act back to the vascular wall in a paracrine fashion to suppress vascular oxidative stress. These findings describe novel, reciprocal interactions between the human vascular wall and perivascular adipose tissue with potentially important clinical implications.
Interactions Between Vascular Wall and Perivascular Adipose Tissue Reveal Novel Roles for Adiponectin in the Regulation of Endothelial Nitric Oxide Synthase Function in Human Vessels

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SUPPLEMENTAL MATERIAL
Supplemental methods

Assessment of Pre-Operative Endothelial Function

FMD and endothelium-independent vasodilatations of the brachial artery were measured the day before surgery by using a linear array transducer, and automated off-line analysis (Vascular Analyser, Medical Imaging Applications LLC), as previously described by our group.\textsuperscript{1} Briefly, brachial artery diameter was recorded before, and sixty seconds after a five minutes forearm blood flow occlusion. A further measurement was made three minutes after a sublingual spray of glycercly trinitrate (400 µg). FMD and endothelium-independent dilatation (EID) of the brachial artery were defined as the %change in vessel diameter after forearm ischaemia or sublingual GTN, respectively.

Blood Sampling and Adiponectin Measurements

Venous blood samples were obtained after 8 hours of fasting, on the morning of surgery. After centrifugation at 2000 g at 4°C for 15 min, plasma or serum was collected and stored at \(-80\) °C until assayed. Whole blood was also collected for genotyping. Serum adiponectin was measured by enzyme linked immunosorbent assay (BioVendor, Brno, Czech Republic).

DNA Extraction and Genotyping

Genomic DNA was extracted from whole blood using standard methods (QIAamp DNA blood Midi kit, Qiagen). Genotyping for the rs17366568 (functional polymorphism in \textit{ADIPOQ} gene) and rs266717 SNPs (functional polymorphism in \textit{ADIPOQ} gene promoter) was performed using TaqMan probes (Applied Biosystems; Assay IDs: C-33187752-10 and...
C-8288442-10 respectively). The assay was run according to manufacturer’s conditions, on an ABI StepOne Plus PCR system.

**Vessel Harvesting**

IMA and SV samples were harvested with a “no touch” technique with their perivascular tissue (peri-IMA-AT and peri-SV-AT) at the time of CABG as we have described previously.15,16 Vascular segments were transferred into oxygenated (95%O₂/ 5% CO₂) ice-cold Krebs Hensleit buffer and the vessel lumen was flushed gently by using an insulin syringe to remove blood. Each vessel was separated from its adipose tissue in the lab, under magnification by the same operator, to limit the between-patients variability. The same anaesthetics were used in all cases, and each sample was always obtained at the same stage of the operation, to limit the between-patients variability.

**Vasomotor Studies**

Vasomotor studies were performed in SV segments obtained during CABG as we have previously described.2,3 Vessels were equilibrated in the organ bath for 60 minutes, in oxygenated (95% O₂/5% CO₂) Krebs-Hensleit buffer at 37°C to achieve a resting tension of 3g. Contractile responses were tested by exposure to Krebs-Hensleit buffer containing potassium chloride (60mM). Four rings from each vessel were pre-contracted with phenylephrine (3x10⁻⁶M); then endothelium-dependent relaxations were quantified using acetylcholine (ACh, 10⁻⁹M to 10⁻⁶M). Finally, relaxations to the endothelium-independent NO donor sodium nitroprusside (SNP, 10⁻¹⁰M to 10⁻⁷M), were evaluated in the presence of the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME; 100μM), as we have previously described.2,3
**Vascular Superoxide Measurements**

Vascular O\(_2^–\) production was measured in fresh, intact IMA and SV segments by using lucigenin (5 μmol/L)-enhanced chemiluminescence, as we have described previously.\(^4\,15\,16\) Vessels were opened longitudinally to expose the endothelial surface and equilibrated for 20 minutes in oxygenated (95% O\(_2\)/5% CO\(_2\)) Krebs-HEPES buffer (pH 7.4) at 37°C. The contribution of uncoupled nitric oxide synthase (NOS) to vascular O\(_2^–\) production was quantified as the change of O\(_2^–\) from baseline to 20 minutes after incubation with the NOS inhibitor L-NAME (100 μmol/L), and presented as delta-LNAME O\(_2^–\) as we have previously described.\(^4\,15\,16\)

**Adipose Tissue Culture**

Samples of Sc-AT, Ms-AT and Peri-SV-AT obtained from patients in study 1, were used to estimate AdN’s biosynthetic rate, in an ex vivo bioassay. Briefly, adipose tissue was isolated after removing the adventitia and washed in sterile phosphate buffer saline. Samples of ~500mg tissue of each type were transferred to the lab within 30 minutes of harvesting. The samples were then cut in ~1-2mm\(^3\) cubes, washed and equilibrated for 1h at 37 °C in Medium-199 containing HEPES 25 mM, gentamycin 50 μg/ml and fatty acid-free bovine serum albumin 1%, in the presence of protease inhibitor (Roche Applied Science, Indianapolis, IN) in a cell culture incubator with 5% CO\(_2\) atmosphere. At the end of the equilibration period the media was changed (1 ml per 200mg tissue) and incubated for 4 hours under the same conditions, as previously described.\(^5\) At 4h, AT culture supernatants were collected, filtered and stored at -80°C until analysis. This allowed the generation of a unique bio-resource of adipose tissue culture supernatants. AdN levels were then quantified in AT culture supernatants by using a high sensitivity ELISA kit (BioVendor).

**RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)**
Samples of Sc-AT, Ms-AT, Peri-SV-AT and Peri-IMA-AT were snap frozen in QIAzol (Qiagen, Stanford, CA) and stored at -80°C until processed. RNA was extracted by using the RNeasy Micro or Mini kit (Qiagen). Ribonucleic acid was reverse transcribed (Quantitect Reverse Transcription kit - Qiagen), and used for qPCR using TaqMan probes (Applied Biosystems, Foster City, CA; Assay IDs ADIPOQ: Hs00605917_m1; PPIA: Hs04194521_s1; PPAR-γ: Hs01115513_m1, CD36: Hs01567185_m1). The reactions were performed in triplicate in 384-well plates, using 5 ng of cDNA per reaction, on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The efficiency of the reaction in each plate was determined based on the slope of the standard curve; relative expression of AdN was calculated using the Pfaffl method, with PPIA (cyclophilin) as housekeeping gene.

**Measurement of Vascular Biopterins**

Vascular BH4, dihydrobiopterin (BH2), and biopterin (B) levels were each determined separately from the same sample, using high-performance liquid chromatography followed by serial electrochemical and fluorescent detection, as we have previously described. Total biopterin levels are the result of the sum of BH4, BH2, and B individual levels. Biopterin levels were expressed as pmol/g in vascular tissue.

**Population in Study 2 and Experimental Procedures**

To examine the direct effects of AdN on the mechanisms regulating vascular NO bioavailability and O₂⁻ production in human vessels, we used a well validated *ex vivo* model of human SVs and IMAs, as we described in the past. For these experiments we recruited 46 patients undergoing CABG following the same exclusion criteria as for study 1. Briefly, serial rings from the same vessel were incubated in oxygenated (95% O₂/5% CO₂) Krebs-HEPES Buffer in the presence or absence of recombinant full-length AdN 10 μg/ml (BioVendor) for 6 hours. The effect of AdN on vascular O₂⁻ (resting and LNAME-inhibitable
O$_2^-$ was quantified by lucigenin (5 μmol/L) enhanced chemiluminescence (as described above) and visualized by oxidative fluorescent dye dihydroethidium (DHE) staining (as described below). The changes in Akt and eNOS phosphorylation status were determined by western blotting. Vascular biopterins (BH4, BH2 and B) were quantified by HPLC, as described above. In additional studies, samples from these patients were incubated with full-length AdN 10 μg/ml in the presence and absence of wortmannin (100 nmol/L) to inhibit PI3 kinase/Akt signaling or 2,4-diamino-6-hydroxypyrimidine (DAHP; 1 mmol/L) to inhibit GTP cyclohydrolase, the rate-limiting enzyme of BH4 biosynthesis, as indicated in the text.

To prove that endogenous oxidative stress regulates 4-HNE generation in vascular tissue, the 4-HNE protein adducts content of human SVs (n=11) and IMAs (n=18) was determined by using western blotting, and linked with vascular O$_2^-$ production in these vessels.

**Ex Vivo Incubation Of Perivascular Adipose Tissue With 4-HNE**

To clarify the role of oxidative stress in the regulation of ADIPOQ gene expression in adipose tissue, samples of Peri-SV and Peri-IMA AT were exposed to the lipid peroxidation product 4-hydroxynonenal (4HNE, 30 μmol/L) in the presence or absence of the PPAR-γ inhibitor T0070907 (10 μmol/L – for peri-SV AT only, due to limited availability of peri-IMA AT). Briefly, samples were collected as described above, separated into three (peri-SV AT) or two (Peri-IMA-AT) parts, each part was then cut into small pieces (~1mm$^3$) and allowed to equilibrate for 2h in medium-199 containing HEPES 25 mM, gentamycin 50 μg/ml and fatty acid-free bovine serum albumin 1%, in the presence of protease inhibitor (Roche Applied Science, Indianapolis, IN) in a cell culture incubator with 5% CO$_2$ atmosphere, as described above. One of the 3 peri-SV AT pieces was exposed to T0070907 during the equilibration period (2h), in order to inhibit PPAR- γ activity. Then, medium was changed and the samples exposed to 4HNE alone or 4HNE+T0070907 (for Peri-SV AT) for
16 hours. At the end of the incubation period, samples were snap frozen for gene expression studies, as described above.

**Oxidative Fluorescent Microtopography**

In situ O$_2^-$ production was determined in vessel cryosections with oxidative fluorescent dye dihydroethidium (DHE), as previously described.$^{1,8}$ Serial SV and IMA rings were incubated with/without AdN 10μg/ml for 6 hours, and they were snap frozen in OCT. Cryosections (30μm) were incubated with DHE (2μmol/L for 5 minutes) in Krebs-Hepes buffer, with or without L-NAME (100μmol/L). Fluorescence images of the endothelium (x63, Zeiss LSM 510 META laser scanning confocal microscope) were obtained from each vessel quadrant. In each case, segments of vessel rings (± L-NAME) were analysed in parallel with identical imaging parameters in a blinded fashion.

**Western Blots For Vascular Akt/p-Akt, eNOS/p-eNOS and 4HNE Protein Adducts**

To examine the direct effects of AdN on Akt phosphorylation at Ser473 and eNOS phosphorylation at Ser1177, as well as quantify the levels of 4-HNE adducts, vascular tissue samples (SVs and IMAs) were homogenized for 30 seconds using a pre-cooled electric homogenizer Polytron in 220 μl of lysis buffer (Invitrogen, UK) or 150μl of RIPA buffer (Cell Signaling Technologies, Danvers, MA) containing a protease inhibitor cocktail (Roche Applied Science). Homogenates were spun at 13,000 rpm for 10 minutes, at 4 °C.

The protein concentration of the supernatants was measured using the BCA™ Protein Assay kit (Pierce, UK). Protein lysates were separated on 4-12% gradient SDS-NuPAGE or 10% Bis-Tris/SDS gel(Invitrogen, UK), and proteins transferred to nitrocellulose membranes (Amersham, UK Ltd.), and blocked with 5% powdered skimmed milk. The membranes were incubated with anti-eNOS antibody (BD Transduction Laboratories, CA), anti-phopho-
eNOS (Ser1177), anti-phospho-Akt (Ser473), anti-pan-Akt (Cell Signaling Technologies) or anti-4HNE antibody (R&D Systems, Minneapolis, USA) as stated. Immunodetection of the primary antibodies was performed with horseradish-peroxidase-conjugated secondary antibodies (Promega) and enhanced chemiluminescence (Amersham Bioscience UK Ltd.) and quantified in relation to the house-keeping protein, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

**Supplemental references**


