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Adiponectin as a Link Between Type 2 Diabetes and Vascular NADPH Oxidase Activity in the Human Arterial Wall: The Regulatory Role of Perivascular Adipose Tissue

Diabetes 2015;64:2207–2219 | DOI: 10.2337/db14-1011

Oxidative stress plays a critical role in the vascular complications of type 2 diabetes. We examined the effect of type 2 diabetes on NADPH oxidase in human vessels and explored the mechanisms of this interaction. Segments of internal mammary arteries (IMAs) with their perivascular adipose tissue (PVAT) and thoracic adipose tissue were obtained from 386 patients undergoing coronary bypass surgery (127 with type 2 diabetes). Type 2 diabetes was strongly correlated with hypoadiponectinemia and increased vascular NADPH oxidase–derived superoxide anions ($O_2^{\cdot-}$). The genetic variability of the *ADIPOQ* gene and circulating adiponectin (but not interleukin-6) were independent predictors of NADPH oxidase–derived $O_2^{\cdot-}$. However, adiponectin expression in PVAT was positively correlated with vascular NADPH oxidase–derived $O_2^{\cdot-}$. Recombinant adiponectin directly inhibited NADPH oxidase in human arteries ex vivo by preventing the activation/membrane translocation of Rac1 and downregulating $p22^{phox}$ through a phosphoinositide 3-kinase/Akt-mediated mechanism. In ex vivo coincubation models of IMA/PVAT, the activation of arterial NADPH oxidase triggered a peroxisome proliferator–activated receptor-γ–mediated upregulation of the adiponectin gene in the neighboring PVAT via the release of vascular oxidation products. We demonstrate for the first time in humans that reduced adiponectin levels in individuals with type 2 diabetes stimulates vascular NADPH oxidase, while PVAT "senses" the increased NADPH oxidase activity in the underlying vessel and responds by upregulating adiponectin gene expression. This PVAT-vessel interaction is identified as a novel therapeutic target for the prevention of vascular complications of type 2 diabetes.

NADPH oxidase, a potent source of superoxide anions ($O_2^{\cdot-}$) in the vascular wall (1), is directly implicated in atherogenesis (2–4). The presence of type 2 diabetes has been related to increased activity of NADPH oxidase in the vascular wall, which is considered to be a key feature in the vascular complications of type 2 diabetes (5). Although NADPH oxidase is partly inhibited by pharmacological interventions (6), the endogenous mechanisms regulating its enzymatic activity in the human arterial wall in individuals with type 2 diabetes are unclear.

Adipose tissue releases both proinflammatory (e.g., interleukin-6 [IL-6]) and anti-inflammatory (e.g., adiponectin) vasoactive molecules (7). Adiponectin is an important adipokine with anti-inflammatory and insulin-sensitizing effects (8), the circulating levels of which are reduced in individuals with type 2 diabetes and obesity (7). While some studies support a causal link between low adiponectin

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Received 3 July 2014 and accepted 20 December 2014.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db14-1011/-/DC1.

A.S.A. and M.M. contributed equally to this study.

See accompanying article, p. 1904.

levels and impaired glucose tolerance (9), others have failed to find such evidence (10). Low circulating levels of adiponectin are associated with increased cardiovascular risk in healthy individuals (11), although recent studies (12) suggest that in advanced cardiovascular disease states the level of circulating adiponectin is increased as a "stress hormone," and its levels become predictive of adverse clinical outcome. On the other hand, proinflammatory cytokines released from human adipose tissue have well-established proatherogenic potential, and they also predict adverse clinical outcome in advanced cardiovascular disease states (13).

The balance between proinflammatory and anti-inflammatory adipokine production in human adipose tissue shows significant regional variability; subcutaneous fat produces more anti-inflammatory, insulin-sensitizing adipokines, while visceral fat produces predominantly proinflammatory adipokines (7). Perivascular adipose tissue (PVAT) may play a key role in vascular physiology, as bioactive molecules released from it could have direct paracrine effects on the underlying vessel (7). Indeed, there is evidence that adiponectin released from PVAT surrounding the human small arteries may have anticontractile effects on the underlying vessels (14), while it also improves endothelial nitric oxide synthase (eNOS) coupling (15). On the other hand, PVAT may play a role in the regulation of vascular oxidative stress and the development of vascular complications in individuals with type 2 diabetes (16).

In the current study, we define the role of type 2 diabetes in the regulation of the vascular redox state, and focus on its impact on NADPH oxidase in the human vascular wall. Then we explore the role of adiponectin and IL-6 as links between type 2 diabetes and vascular oxidative stress; we investigate, for the first time in humans, the mechanisms by which hypoadiponectinemia affects vascular NADPH oxidase activity and explore the cross-talk between vascular NADPH oxidase and peroxisome proliferator–activated receptor-γ (PPAR-γ) signaling in the control of adiponectin expression in human PVAT.

RESEARCH DESIGN AND METHODS

Population and Protocol

The population of study 1 consisted of 386 patients (Table 1) undergoing elective coronary artery bypass grafting (CABG) surgery. Exclusion criteria were any inflammatory, infectious, liver, or renal disease or malignancy. Patients with heart failure or those receiving nonsteroidal anti-inflammatory drugs, dietary supplements, or antioxidant vitamins were also excluded from the study. Blood samples were obtained on the morning of the surgery. During CABG surgery, internal mammary artery (IMA) segments were harvested by preserving their PVAT. In addition to the PVAT surrounding the IMA, samples of thoracic adipose tissue (Th-AT) (not in proximity with any visible vessel) were also harvested as "control" samples to the PVAT. Adipose tissue samples from all sites were snap frozen for gene expression studies while samples of Th-AT were also cultured ex vivo for 4 h to quantify the release of adiponectin and IL-6. In study 2, we included 67 additional patients undergoing CABG surgery (using the same inclusion criteria), and IMA/PVAT samples were used for ex vivo experiments, as described below. The study was approved by the Research Ethics Committee, and the subjects gave written informed consent.

Table 1—Demographic characteristics of study participants

Participants	Clinical studies (study 1) (n = 386)	Ex vivo studies (study 2) (n = 67)
Age (years)	65.8 ± 0.5	65.5 ± 1.3
Male sex	83.7	97.0
Hypertension	68.9	76.1
Hyperlipidemia	62.4	77.6
Type 2 diabetes	32.9	26.9
Smoking status		
Active	22.8	4.4
Ex-smoker	46.6	59.7
BMI (kg/m^2)	27.7 ± 0.2	29.4 ± 0.5
Cholesterol (mg/dL)	177.3 ± 2.4	146.9 ± 12.4
Glucose (mg/dL)	125.4 ± 3.1	97.2 ± 5.4
Insulin (μU/mL)	15.49 ± 1.43	13.98 ± 2.01
HOMA-IR	5.39 ± 0.54	3.36 ± 0.03
HDL (mg/dL)	38.8 ± 0.6	42.5 ± 7.7
Triglycerides (mg/dL)	141.4 ± 4.9	149.7 ± 40.7
Hemoglobin (g/dL)	13.3 ± 0.1	13.7 ± 0.2
White blood cell count (cells/μL)	7,288 ± 151	8,553 ± 749
Platelet count (cells/μL)	239,782 ± 4,224	225,900 ± 8,100
Creatinine (mg/dL)	1.07 ± 0.02	0.94 ± 0.04
rs266717 alleles		
CC	23.2	
CT	48.1	
TT	28.7	
rs17366568 alleles		
AA	1.3	
AG	18.5	
GG	80.2	
Medication		
ACEi	58.5	46.2
ARB	9.8	16.4
β-Blocker	75.3	88.0
Aspirin/clopidogrel	82.1	93.6
Statins	78.2	88.0
CCB	24.9	41.7

Data are reported as the mean ± SEM or %. ACEi, ACE inhibitor; ARB, angiotensin receptor blocker; CCB, calcium channel blocker.

Measurements of Circulating Biomarkers

Serum levels of total adiponectin, IL-6, and high molecular weight (HMW) adiponectin were measured by ELISA (BioVendor, Brno, Czech Republic; R&D Systems; and Otsuka, respectively). Plasma malondialdehyde (MDA), a marker of systemic oxidative stress, was quantified by using the thiobarbituric acid reactive substances fluorometric assay,

as previously described (17). Plasma 4-hydroxynonenal (4-HNE [a product of lipid peroxidation]) was measured by ELISA (MyBioSource, San Diego, CA). Serum insulin levels were measured by chemiluminescent microparticle immunoassay, and serum glucose levels were measured by the hexokinase method using commercial kits (Abbott, Wiesbaden, Germany). HOMA of insulin resistance (IR) was calculated by using the formula (glucose × insulin)/405, with glucose measured in milligrams per deciliter and insulin in milliunits per liter (18).

DNA Extraction and Genotyping

Genomic DNA was extracted from whole blood using commercial kits (QIAGEN, Stanford, CA), and genotyping for the rs17366568 (functional polymorphism in *ADIPOQ* gene, which encodes for adiponectin) and rs266717 (functional polymorphism in *ADIPOQ* gene promoter) was performed by using TaqMan probes (Life Technologies). These two functional single nucleotide polymorphisms (SNPs) have shown an effect on adiponectin levels in recent genome-wide association studies (19).

Vascular O2$^{\cdot -}$ Measurements

Vascular $O_2^{\cdot -}$ production was measured in fresh, intact IMA segments by using lucigenin-enhanced chemiluminescence, as previously described (20). NADPH oxidase activity was estimated by quantifying the NADPH-stimulated $O_2^{\cdot -}$ in these vessels, and the specificity of this measurement was assessed by using the specific NADPH oxidase inhibitor Vas2870. In these experiments, peripheral blood mononuclear cells were used as a positive control. Previous observations from our group have yielded a very strong correlation between NADPH-stimulated $O_2^{\cdot -}$ in intact and homogenized vessels ($r = 0.75$, $P < 0.001$), suggesting that the use of intact human vessels provides a more "physiological" alternative to homogenates.

Adipose Tissue Culture

Samples of Th-AT obtained from patients in study 1 were used to estimate the biosynthetic rate of adiponectin in an ex vivo bioassay, as we have previously described (15). Each adipose tissue sample was cultured for 4 h. The secretion of total adiponectin, IL-6, and HMW adiponectin in adipose tissue culture supernatants was measured by ELISA (BioVendor; R&D Systems; and Otsuka, respectively).

RNA Isolation and Quantitative Real-Time PCR

RNA was extracted from adipose tissue or IMA segments and reverse transcribed by commercially available kits (QIAGEN). The expression of *ADIPOQ*, *IL-6*, and *PPAR-γ* in adipose tissue and *AdipoR1*, *AdipoR2*, and *CDH13* in adipose tissue and IMA were quantified with the Pfaffl method by quantitative real-time PCR (TaqMan Probes; Life Technologies), using PPIA (cyclophilin) or GAPDH, respectively, as the housekeeping gene.

Study of the Direct Effects of Adiponectin on NADPH Oxidase Ex Vivo

To examine the direct effects of adiponectin on NADPH oxidase activity in human IMA segments, we used a well-validated ex vivo model of human vessels that we have previously described (20). For these experiments, we recruited 67 additional patients undergoing CABG surgery following the same exclusion criteria as for study 1. Briefly, serial rings from the same vessel were incubated in oxygenated ($95\%O_2/5\%CO_2$) Krebs-HEPES buffer in the presence or absence of recombinant full-length adiponectin 10 μg/mL (RD172029100; BioVendor) for 6 or 18 h, as stated. The effect of adiponectin on NADPH-stimulated $O_2^{\cdot -}$ was quantified by lucigenin chemiluminescence (as described above). To accurately estimate the effect of adiponectin on NADPH oxidase activity, the specific NADPH oxidase inhibitor Vas2870 (40 μmol/L; Sigma-Aldrich) was used and the Vas2870-inhibitable signal was quantified. Given that previous cell culture studies suggested that the effect of adiponectin on vascular cells is mediated via phosphoinositide 3-kinase (PI3K)/Akt signaling, in some experiments the IMA segments were also incubated with wortmannin (100 nmol/L; Sigma-Aldrich), inhibitor of the PI3K/Akt signaling, as described below.

Oxidative Fluorescent Microtopography

In situ $O_2^{\cdot -}$ production was determined in vessel cryosections with the oxidative fluorescent dye dihydroethidium, as previously described (20). Serial IMA rings were incubated with and without adiponectin 10 μg/mL for 6 h. NADPH oxidase inhibitor (Vas2870) was used to determine the contribution of this enzyme to the observed signal.

Measurement of Vascular Rac1 Activation and Membrane Translocation of Rac1 and p47^{phox}

Rac1 activation was evaluated by a commercially available affinity precipitation assay using the PAK1-PBD–conjugated glutathione agarose beads (Millipore, Temecula, CA) (6). To estimate the membrane translocation of Rac1 and p47^{phox}, we performed differential centrifugation for the isolation of membrane proteins, and membrane-translocated Rac1 or p47^{phox} protein was determined by Western immunoblotting, as previously described (6).

Western Blots

Western immunoblotting was used to examine the direct effects of adiponectin on NOX1, NOX2, and NOX4 (Abcam, Cambridge, U.K.); p47phox, p67phox, phospho-Akt (Ser473), and pan-Akt (Cell Signaling Technology, Danvers, MA); and total Rac1 (Merck Millipore, Billerica, MA) expression in serial IMA segments, as well as evaluate the content of 4-HNE (Abcam) and MDA (Sigma) protein adducts in IMA samples that were obtained from patients with and without type 2 diabetes, exhibiting high/low NADPH-stimulated $O_2^{\cdot -}$ generation.

Total Antioxidant Capacity of Human IMAs

To investigate differences in the total antioxidant capacity of IMAs from patients with and without type 2 diabetes,

we used a commercially available kit (Cell Biolabs, San Diego, CA). This kit quantifies the Cu^{2+}-reducing equivalent per milligram of protein as an index of the overall antioxidant capacity of the tissue.

Cocultures of Human IMA and PVAT: Examining the Effect of Vascular NADPH Oxidase on Adiponectin Expression in PVAT

To investigate the influence of vascular oxidative stress on adiponectin expression in PVAT, we performed coincubations of human IMA with and without their respective PVAT. Briefly, PVAT was incubated in a weight-adjusted volume of modified Medium-199 for 18 h either alone or with NADPH (100 μmol/L) or with its respective IMA tissue or with its IMA tissue plus 100 μmol/L NADPH (to stimulate NADPH oxidase) or with its IMA tissue plus 100 μmol/L NADPH plus 300 units/mL polyethylene glycol-superoxide dismutase (PEG-SOD) (to scavenge $O_2^{\cdot -}$). *ADIPOQ* gene expression was determined in the PVAT samples as described above.

Ex Vivo Incubation of Human PVAT With 4-HNE/MDA

To investigate the effects of lipid oxidation on *ADIPOQ* gene expression in PVAT, samples of human PVAT were exposed ex vivo to 4-HNE (30 μmol/L) or MDA (1 mmol/L) for 16 h in the presence or absence of PPAR-γ activity inhibitor T0070907 (10 μmol/L), and their effects on *ADIPOQ* and *PPAR-γ* gene expression were measured, as described above.

Statistical Analysis

Continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test, and non-normally distributed variables were log transformed for analysis to achieve normality.

Sample size calculations were based on previous data from our laboratory. For the clinical studies, we estimated that a total number of 300 patients would allow us to detect a 10% difference in log(NADPH-stimulated $O_2^{\cdot -}$) in IMA segments between patients in the two extreme tertiles, assuming an SD of 0.38, α of 0.05, and power of 90%. For the ex vivo experiments, sample size calculations were performed based on our previous experience with this model (6), and we estimated that with $n = 5$ pairs of samples (serial rings from the same vessel) we would be able to identify a 20% change of log(NADPH-stimulated $O_2^{\cdot -}$) with α of 0.05, power of 90%, and an SD for a difference in the response of the pairs of 0.2.

In the clinical studies, continuous variables among three groups were compared by using one-way ANOVA, while comparisons between two groups were performed by unpaired *t* tests, corrected by using the Bonferroni post hoc correction for multiple testing when more than one comparison was performed. Categorical variables were compared by using the χ^2 test, as appropriate. Correlations between continuous variables were assessed by calculating the Pearson or Spearman correlation coefficient as stated. For the ex vivo experiments (where serial rings from the same vessel were incubated with 0 or 10 μg/mL adiponectin), we performed a repeated-measures ANOVA followed by a paired-samples *t* test for individual comparisons with a Bonferroni correction when there were multiple tests, as appropriate. A Wilcoxon paired-rank test followed by Bonferroni correction was used to compare values expressed as fold changes versus the control.

Linear regression was performed by using log(NADPH-stimulated $O_2^{\cdot -}$) as the dependent variable. As independent variables, we used log(serum adiponectin) or the *ADIPOQ* genotype plus those of the clinical demographic characteristics (age, sex, type 2 diabetes, smoking, dyslipidemia, and hypertension) that showed an 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 model. All statistical tests were performed by using SPSS version 20.0, and $P < 0.05$ was considered to be statistically significant.

RESULTS

The patients' demographic characteristics are presented in Table 1. We first demonstrate that IR, as defined by HOMA-IR ≥2.8, and type 2 diabetes are both associated with reduced circulating levels of adiponectin in our cohort (Fig. 1*A*). Similarly, IR and type 2 diabetes were linked to increased NADPH oxidase–derived $O_2^{\cdot -}$ in the human IMA (Fig. 1*B*). There was no significant association between IR or type 2 diabetes and circulating levels of MDA (Fig. 1*C*) or 4-HNE (Fig. 1*D*), both of which are markers of systemic oxidative stress, suggesting that the impact of type 2 diabetes and IR on the vascular redox state cannot be accurately monitored by measuring levels of circulating biomarkers of oxidative stress. There was no significant correlation between plasma MDA and NADPH oxidase–derived $O_2^{\cdot -}$ levels in IMAs from the same patients ($r = 0.076$, $P = 0.448$), suggesting that arterial NADPH oxidase activity is independent of what is conventionally defined as "systemic oxidative stress." We then examined whether circulating adiponectin levels were related to NADPH oxidase activity in these vessels and found that NADPH-stimulated $O_2^{\cdot -}$ levels were lower in patients with high levels of circulating adiponectin (Fig. 2*A*). In contrast, the serum IL-6 level was not related to the NADPH-stimulated $O_2^{\cdot -}$ level in the IMA (Fig. 2*B*). These results imply that the increased NADPH oxidase activity observed in IR and type 2 diabetes could be caused, at least in part, by reduced circulating levels of adiponectin. To explore this concept, and in an attempt to establish the direction of the observed association among IR and type 2 diabetes, adiponectin levels, and arterial NADPH oxidase activity, we then tested whether the genetic variability of the *ADIPOQ* gene encoding adiponectin (rs17366568 and rs266717 SNPs) (21,22) affects NADPH oxidase activity in the IMA. Building on the results of previously published genome-wide association studies (21,22), we observed that rs17366568G and rs266717T alleles had an additive effect on both circulating levels of adiponectin (Fig. 2*C*) and adiponectin gene

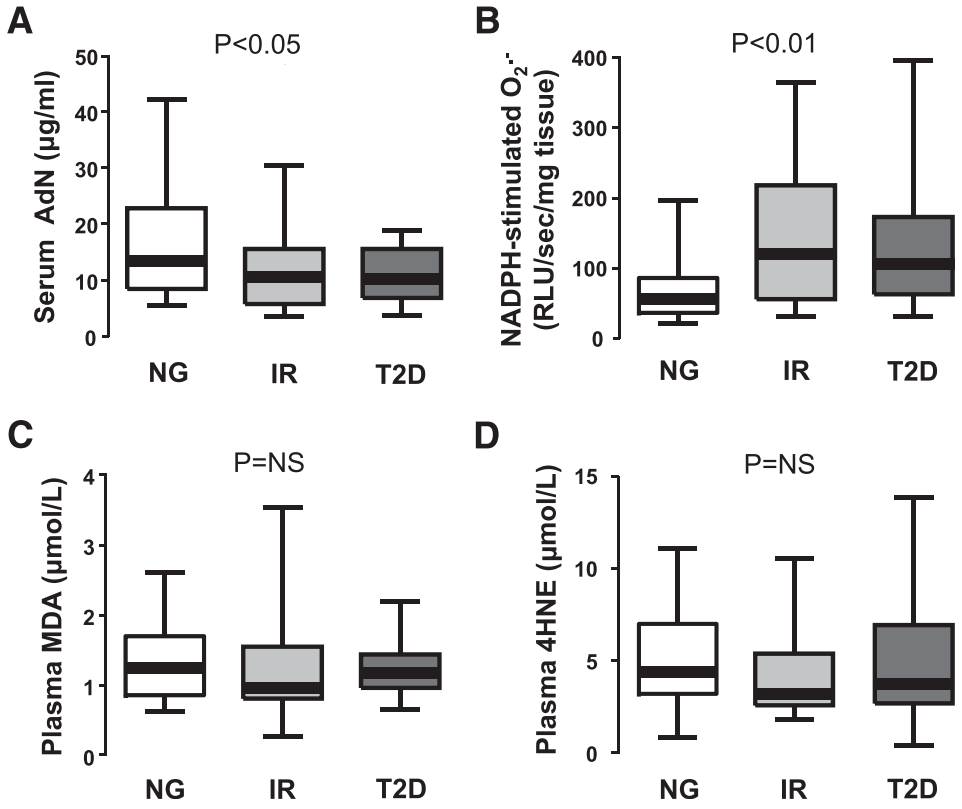

Figure 1—Patients with IR or type 2 diabetes (T2D), compared to normoglycemic (NG) patients, had significantly lower circulating levels of adiponectin (AdN) (panel *A*), as well as increased levels of NADPH oxidase–derived $O_2^{\cdot-}$ in the vascular wall of the IMA (panel *B*). There was no significant association between IR/T2D and plasma MDA (panel *C*) or 4-HNE levels (panel *D*), both of which are markers of systemic oxidative stress. Values are expressed as the median (25th–75th percentile). NS, not significant; RLU, relative light units.

expression in Th-AT (Fig. 2*D*), but not in PVAT (P = NS, data not shown). Importantly, the number of rs17366568G and rs266717T alleles was inversely related to the presence of type 2 diabetes (Spearman $\rho = -0.176$, $P = 0.001$). The number of rs17366568G and rs266717T alleles was also negatively related to NADPH-stimulated $O_2^{\cdot-}$ in IMA segments (Fig. 2*E*), while it had no significant impact on systemic oxidative stress, as estimated by plasma MDA levels (Fig. 2*F*) or systemic inflammation (defined by serum IL-6 levels, data not shown). In univariate analysis, log(NADPH-stimulated $O_2^{\cdot-}$) was related to both log(serum adiponectin) ($r = -0.219$, $P < 0.0001$) and type 2 diabetes ($\rho = 0.195$, $P = 0.001$) but also to smoking status ($\rho = 0.172$, $P = 0.003$) and hypertension ($\rho = 0.149$, $P = 0.009$). To correct the association between adiponectin and type 2 diabetes and NADPH oxidase activity for the confounding effects of smoking and hypertension, we performed a multivariable analysis in which we confirmed that in human arteries log(NADPH-stimulated $O_2^{\cdot-}$) is related to log(serum adiponectin) (β [SE] −0.233 [0.074], $P = 0.002$) and type 2 diabetes (0.112 [0.047], $P = 0.017$) independently of hypertension (0.108 [0.048], $P = 0.026$) and smoking (0.088 [0.031], $P = 0.004$), with an R^2 for the model of 0.112. When the additive model of the two *ADIPOQ* SNPs was included into the multivariable analysis, the predictive value of the model was slightly improved ($R^2 = 0.136$), and the number of rs17366568G and rs266717T alleles was a predictor of log(NADPH-stimulated $O_2^{\cdot-}$) (β [SE] −0.081 [0.025], $P = 0.001$) independently of hypertension (0.150 [0.053], $P = 0.005$) and smoking (0.091 [0.033], $P = 0.006$). Interestingly, type 2 diabetes lost its predictive value for vascular NADPH oxidase activity in this model (β [SE] 0.068 [0.052], $P = 0.192$). These findings suggest that circulating levels of adiponectin (but not of IL-6) might be a regulator of NADPH oxidase activity in the human arterial wall and interrelated with diabetes in a way that could partly mediate the effects of type 2 diabetes on vascular NADPH oxidase activity.

We then examined whether adiponectin release and *ADIPOQ* gene expression in Th-AT correlate with NADPH oxidase activity in the human arteries. We observed that both gene expression (Fig. 3*A*) and the release of total (Fig. 3*B*) or HMW (Supplementary Fig. 1) adiponectin from Th-AT were inversely correlated with levels of NADPH-stimulated $O_2^{\cdot-}$ in these vessels. Paradoxically, *ADIPOQ* gene expression from PVAT was positively correlated with NADPH oxidase–derived $O_2^{\cdot-}$ in the underlying arterial wall (Fig. 3*C*), implying that adiponectin expression in PVAT may be regulated by NADPH oxidase in the underlying vessel, possibly through the release of a paracrine signal from the

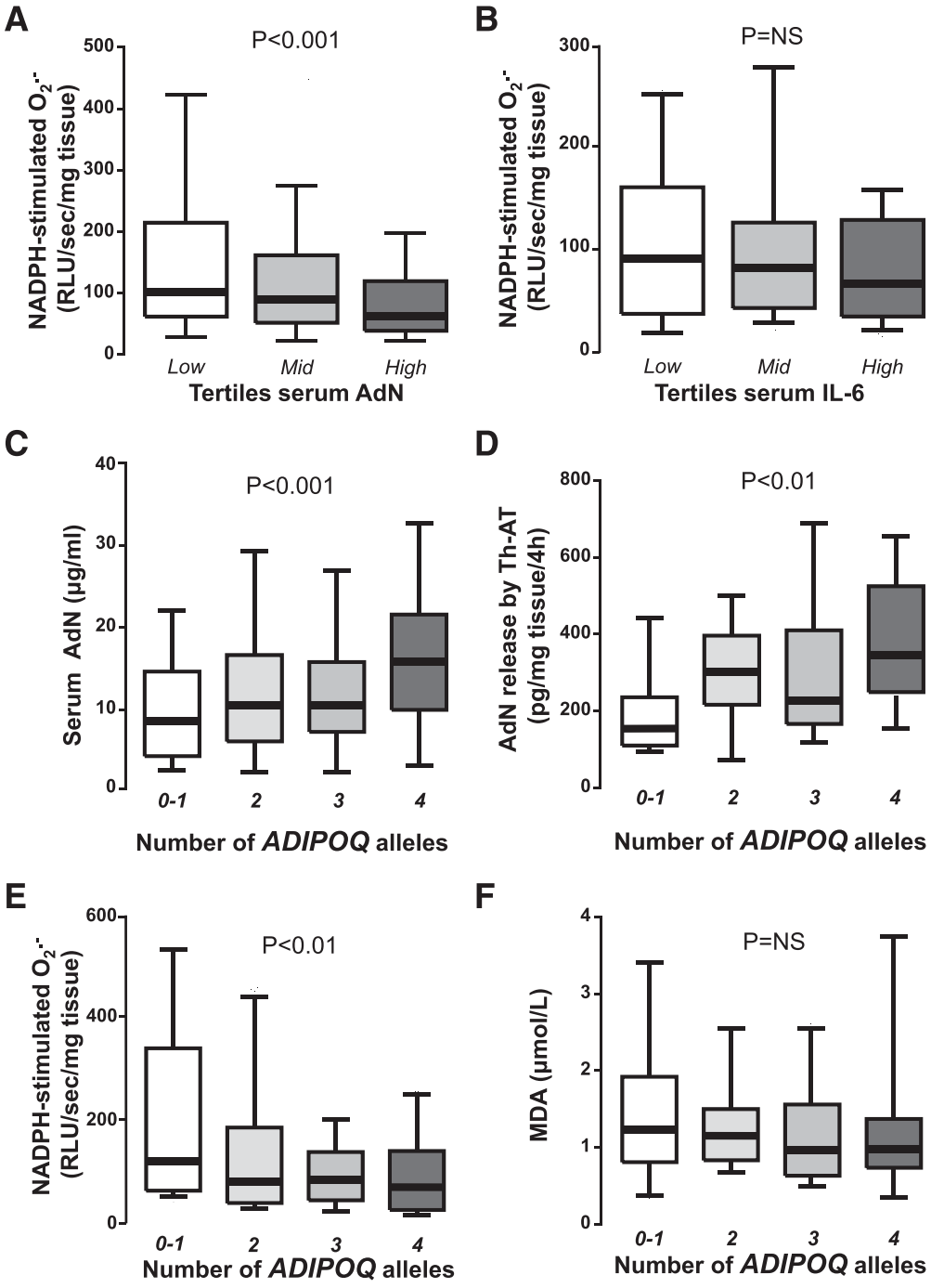

Figure 2—Patients with high serum levels of adiponectin (AdN) had significantly lower NADPH-stimulated $O_2^{\cdot-}$ generation in the vascular wall of their left IMA (panel *A*), while serum IL-6 levels were unrelated to NADPH-stimulated $O_2^{\cdot-}$ levels in these vessels (panel *B*). We confirmed that the total number of rs17366568G alleles (polymorphism in *ADIPOQ* gene) and rs266717T alleles (polymorphism in *ADIPOQ* promoter region) had an additive effect on circulating levels of adiponectin (panel *C*) and its release from Th-AT after 4 h of tissue culture (panel *D*). By using this genetic model, we observed that the number of rs17366568G/rs266717T alleles was inversely related to NADPH-stimulated $O_2^{\cdot-}$ levels in the arterial wall (panel *E*). However, the genetic variability of the *ADIPOQ* gene had no effect on plasma MDA levels, a marker of systemic lipid peroxidation (panel *F*). Values are expressed as the median (25th–75th percentile). NS, not significant; RLU, relative light units.

vessel to its PVAT. The ex vivo release of IL-6 by Th-AT as well as *IL-6* gene expression in Th-AT and PVAT were not correlated with NADPH-stimulated $O_2^{\cdot-}$ in the human arteries (Fig. 3*D–F*). There was no significant difference in the expression of *ADIPOQ* or the release of total or HMW adiponectin from paired Th-AT and PVAT (data not shown).

Given that the aforementioned associations do not fully document a causal association between adiponectin

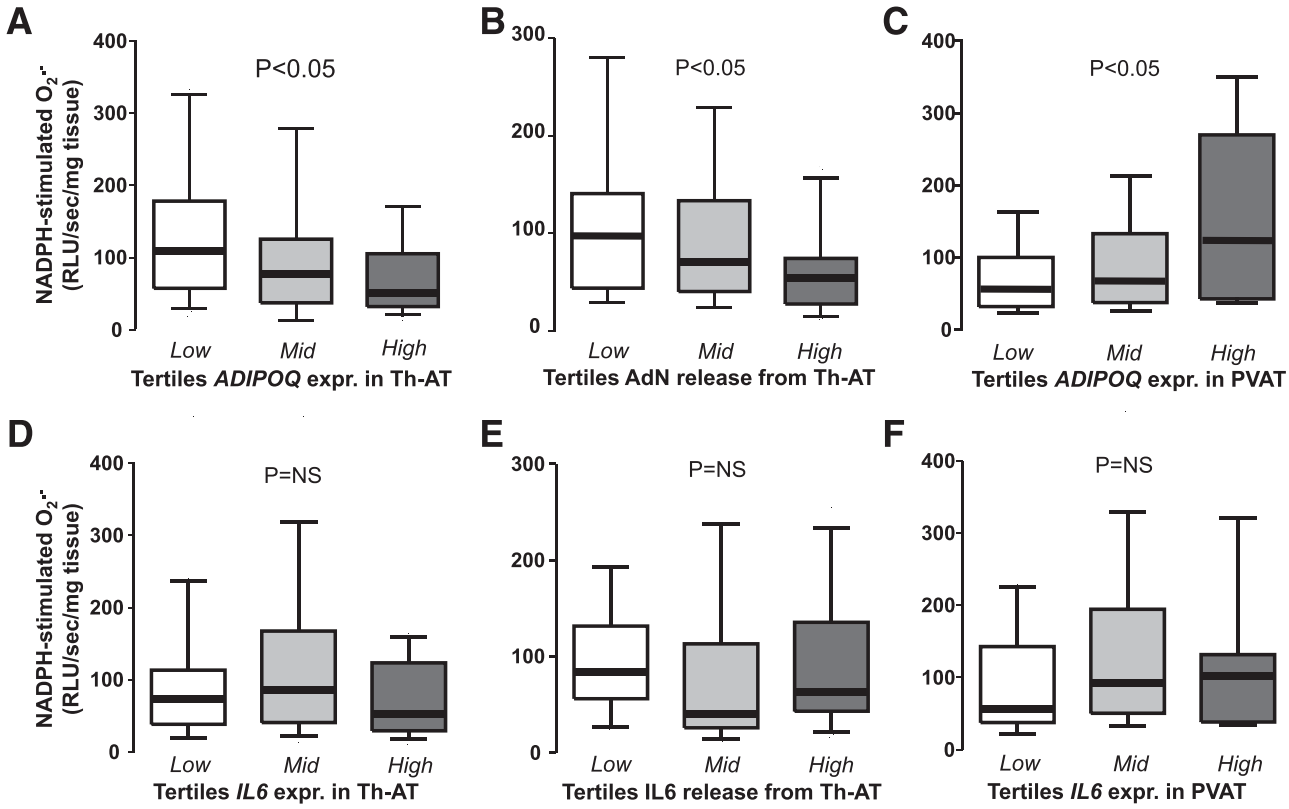

Figure 3—There was an inverse association between NADPH-stimulated $O_2^{\cdot-}$ levels in human IMAs and adiponectin (AdN) gene expression (*ADIPOQ* gene) (panel *A*) and release (after 4 h of tissue culture) (panel *B*) in Th-AT. However, there was a positive association between vascular NADPH-stimulated $O_2^{\cdot-}$ levels and *ADIPOQ* gene expression in the PVAT surrounding these vessels (panel *C*). Importantly, there was no correlation between NADPH-stimulated $O_2^{\cdot-}$ levels in the human arterial wall and IL-6 gene expression (panel *D*) and release (panel *E*) in Th-AT or IL-6 gene expression in PVAT (panel *F*). Values are expressed as the median (25th–75th percentile). expr., expression; NS, not significant; RLU, relative light units; sec., seconds.

and vascular oxidative stress in humans, we performed additional mechanistic ex vivo experiments with serial IMA segments obtained from 67 patients undergoing CABG surgery. In these experiments, incubation of these arterial segments with adiponectin 10 μg/mL, a biologically relevant concentration (11), for 6 h significantly reduced levels of $O_2^{\cdot-}$ and its Vas2870-inhibitable $O_2^{\cdot-}$ fraction in all layers of the vascular wall (Fig. 4). Similarly, the levels of NADPH-stimulated $O_2^{\cdot-}$ (Fig. 5*A*) and its Vas2870-inhibitable fraction (Fig. 5*B*) were significantly reduced by adiponectin, an effect reversed by the PI3K/Akt signaling inhibitor wortmannin.

To further explore the molecular mechanisms by which adiponectin affects NADPH oxidase activity in the human arterial wall, we examined its impact on the expression of NADPH oxidase subunits and Rac1 activation. We observed that the incubation of IMA segments with adiponectin 10 μg/mL for 6 h had no effect on the total protein levels and gene expression of NOX1, NOX2, NOX4, $p47^{phox}$, $p67^{phox}$, and Rac1 (data not shown). On the contrary, there was a significant reduction in $p22^{phox}$ gene expression at 6 h, which was prevented by wortmannin (Fig. 5*C*). This effect was not translated into a reduction in vascular $p22^{phox}$ protein levels at that early time point (Fig. 5*D*), and as such it could not explain the significant and rapid decrease in NADPH oxidase activity observed in these vessels after 6 h of incubation. However, adiponectin rapidly inhibited the activation of Rac1, as evidenced by a reduction in guanosine triphosphate (GTP)–bound Rac1, (Fig. 5*E*), and its translocation to the membrane (Fig. 5*F*), both of which were reversed by wortmannin. These findings suggest that adiponectin suppresses NADPH oxidase activity in the human arterial wall through a PI3K/Akt-mediated inhibition of Rac1 activation.

Given the first observation that $p22^{phox}$ gene expression (but not its protein level) was downregulated in a PI3K/Akt-dependent way after 6 h of incubation of human IMAs with adiponectin, we performed longer-term incubations (for 18 h), where we confirmed that the effect of adiponectin on vascular $O_2^{\cdot-}$ levels in these vessels (Fig. 5*G* and *H*) was now also accompanied by a reduction in the protein level of the vascular $p22^{phox}$ subunit (Fig. 5*I*), documenting a second subacute effect of adiponectin on NADPH oxidase.

To explore the role of adiponectin in vascular NADPH oxidase regulation in individuals with type 2 diabetes, we performed additional experiments in IMAs obtained from patients with and without type 2 diabetes. We observed that type 2 diabetes is related to increased membrane

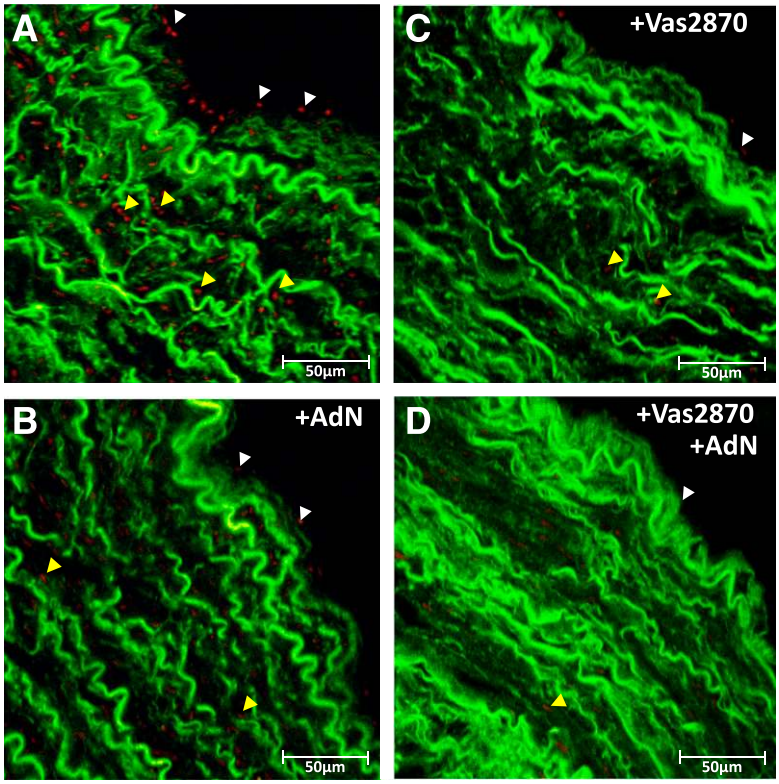

Figure 4—Ex vivo incubation of serial human left IMA segments with adiponectin (AdN) 10 μg/mL for 6 h resulted in significant reduction of $O_2^{\cdot -}$ (red dots) production in both the vascular endothelium (white arrowheads) and the wall (yellow arrowheads) (panels *A* and *B*). Inhibition with Vas2870 (a specific inhibitor of NADPH oxidase) significantly supressed $O_2^{\cdot -}$ production in both the endothelium and the vascular wall (panels *C* and *D*), suggesting that adiponectin suppressed the NADPH oxidase–derived $O_2^{\cdot -}$ signal throughout the entire vascular wall. Images of dihydroethidium staining were viewed by a Zeiss LSM 510 META laser-scanning confocal microscope at ×40 magnification.

translocation of the cytosolic subunits $p47^{phox}$ and Rac1 of NADPH oxidase, explaining the activation of vascular NADPH oxidase in these patients (Fig. 6*A* and *B*). Interestingly, we observed that adiponectin (10 μg/mL for 6 h) reduced NADPH oxidase–derived $O_2^{\cdot -}$ levels in IMAs from patients with or without type 2 diabetes (Fig. 6*C* and *D*). In addition, we did not observe significant differences in the expression of the three major adiponectin receptors (AdipoR1, AdipoR2, and T-cadherin [CDH13]) in IMAs from patients with and without type 2 diabetes (Fig. 6*E*). We also observed lower Akt phosphorylation at Ser473 (an activation site) in IMAs obtained from patients with type 2 diabetes compared with those without type 2 diabetes (Fig. 6*E*), and incubation of these vessels with adiponectin induced phosphorylation at Ser473 and the activation of Akt, irrespective of the presence of type 2 diabetes (Fig. 6*G* and *H*).

To explain the positive association between NADPH-stimulated $O_2^{\cdot -}$ levels in the IMA and *ADIPOQ* gene expression in the surrounding PVAT, we hypothesized that vascular oxidative stress may trigger the expression of the adiponectin gene in PVAT. To test this hypothesis, we performed coincubation experiments of PVAT with and without its respective IMA for 18 h, as well as after stimulation of vascular NADPH oxidase by using NADPH (100 μmol/L) (Fig. 7*A*). We found that removal of the underlying vascular tissue resulted in an ~50% reduction of *ADIPOQ* gene expression in PVAT, whereas stimulation of NADPH oxidase led to even higher levels of adiponectin gene expression in PVAT. This was not due to the direct effects of NADPH on PVAT (e.g., via effects on P2X receptors) (23), because incubation of PVAT plus NADPH without the underlying vessel still led to a reduction in *ADIPOQ* gene expression (Fig. 7*A*). Importantly, scavenging reactive oxygen species with PEG-SOD not only prevented the NADPH-induced upregulation of the *ADIPOQ* gene, but also resulted in its downregulation below the baseline (control) expression levels (PVAT plus IMA). Taken together, these results prove that increased oxidative stress originating from the vessel wall is able to upregulate adiponectin gene expression in the surrounding PVAT.

To explore the mechanisms linking increased vascular NADPH oxidase activity with upregulation of the *ADIPOQ* gene in PVAT, we then examined whether products of oxidation released from the vascular wall in response to increased NADPH oxidase activity (4-HNE or MDA) can modify *ADIPOQ* gene expression via changes in vascular

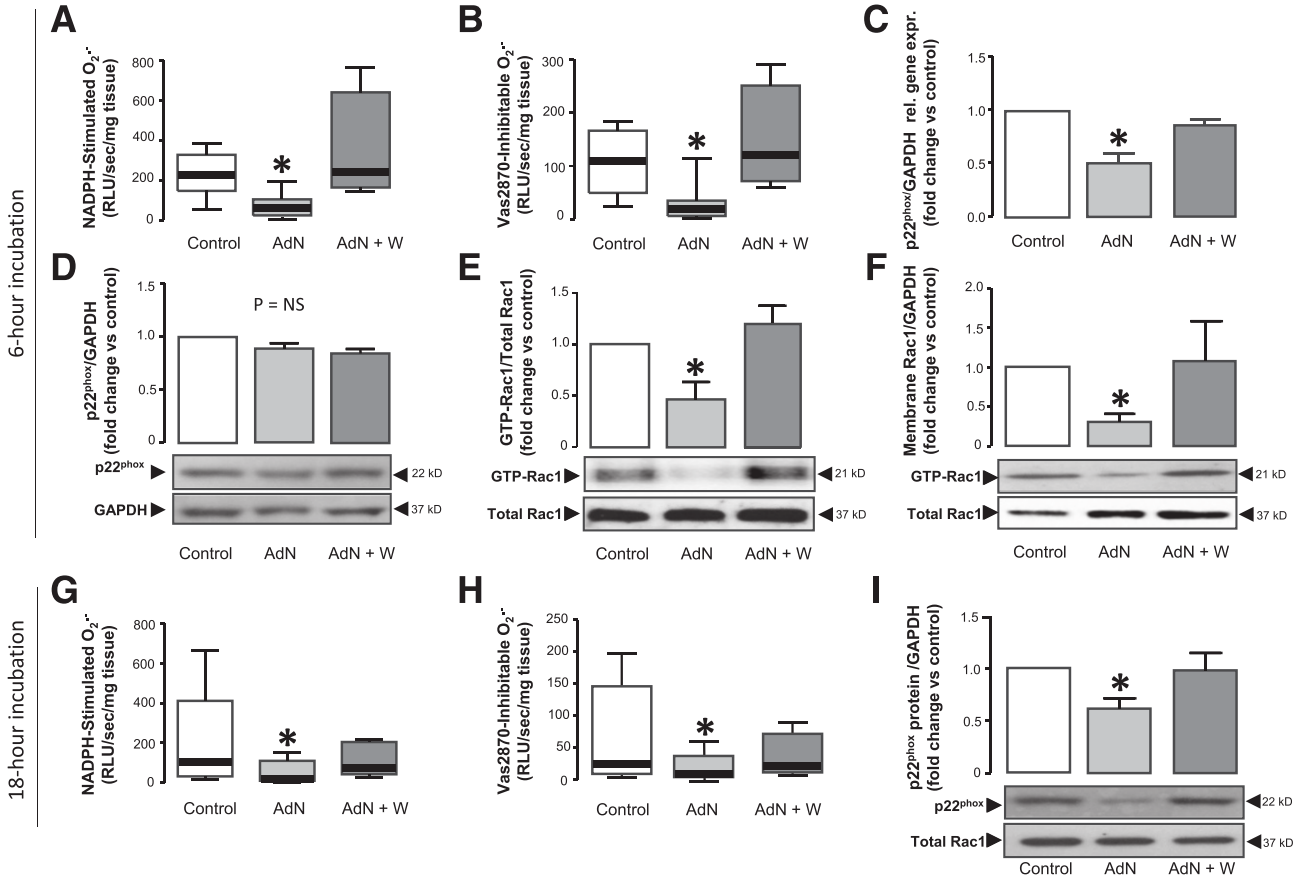

Figure 5—Ex vivo incubation of serial human left IMA rings (n = 10) with adiponectin (AdN) 10 μg/mL for 6 h significantly reduced NADPH-stimulated $O_2^{\cdot-}$ production (panel *A*) and its Vas2870-inhibitable fraction (panel *B*) as measured by lucigenin-enhanced chemiluminescence in fresh tissue samples. This effect was reversed by coincubation with wortmannin (W; 100 nmol/L), an inhibitor of the PI3K/Akt pathway (panels *A* and *B*). Incubation of human IMA segments with adiponectin for 6 h (n = 8) resulted in significant downregulation of the $p22^{phox}$ subunit of NADPH oxidase (panel *C*), although there was no significant reduction of $p22^{phox}$ protein levels (panel *D*) to explain the changes in the overall NADPH oxidase activity. However, adiponectin significantly reduced the membrane translocation (panel *E*) and activation (panel *F*) of Rac1, an effect that was reversed by wortmannin (n = 7). The effect of incubation with adiponectin 10 μg/mL on NADPH-stimulated $O_2^{\cdot-}$ production (panel *G*) and its Vas2870-inhibitable fraction (panel *H*) remained significant after 18 h of ex vivo culture, and this effect was again partly reversed by wortmannin (100 nmol/L) (n = 9). In these experiments, the reduction of $p22^{phox}$ protein levels was significant, and this effect was reversed by wortmannin (panel *I*). Values are presented as the median (25th–75th percentile) (panels *A*, *B*, *G*, and *H*) or the means ± SEM (panels *C–F* and *I*). *$P < 0.05$ vs. control. expr., expression; NS, not significant; RLU, relative light units; sec., seconds.

PPAR-γ signaling in the neighboring PVAT. Increased levels of 4-HNE protein adducts were observed in human IMAs with higher NADPH oxidase activity, confirming that NADPH oxidase–derived $O_2^{\cdot-}$ leads to the production of 4-HNE in these vessels (Fig. 7*B*). Similarly, IMAs obtained from patients with type 2 diabetes had higher levels of these adducts (4-HNE and MDA protein adducts) compared with patients without diabetes (Fig. 7*C* and Supplementary Fig. 2), confirming the increased vascular oxidative stress in individuals with type 2 diabetes. In addition, IMAs from patients with type 2 diabetes also had significantly reduced total antioxidant capacity (Supplementary Fig. 2), suggesting the depletion of endogenous vascular antioxidant systems in individuals with diabetes. These findings lead to the conclusion that type 2 diabetes increases arterial oxidative stress, leading to the local production of oxidation products such as 4-HNE and/or MDA, which may mediate the "reverse inside-to-outside signal" linking vascular oxidative stress with the upregulation of the *ADIPOQ* gene in PVAT.

We then incubated PVAT samples with 4-HNE or MDA for 16 h and found that 4-HNE (but not MDA) upregulated *ADIPOQ* gene expression (by 63% vs. control) (Fig. 7*D* and Supplementary Fig. 2). 4-HNE also upregulated the *PPAR-γ* gene (Fig. 7*E*), while it had no effect on the expression of the *IL-6* gene in the human PVAT (reduced by 10% vs. control, P = 0.66). This effect of 4-HNE on *ADIPOQ* gene expression in PVAT was reversed by T0070907 (a PPAR-γ activity inhibitor), suggesting that PPAR-γ activation is critically involved in this process (Fig. 7*D* and *E*).

DISCUSSION

In the current study, we examine the role of adiponectin as a link between type 2 diabetes and the vascular redox state. We demonstrate that circulating levels of adiponectin

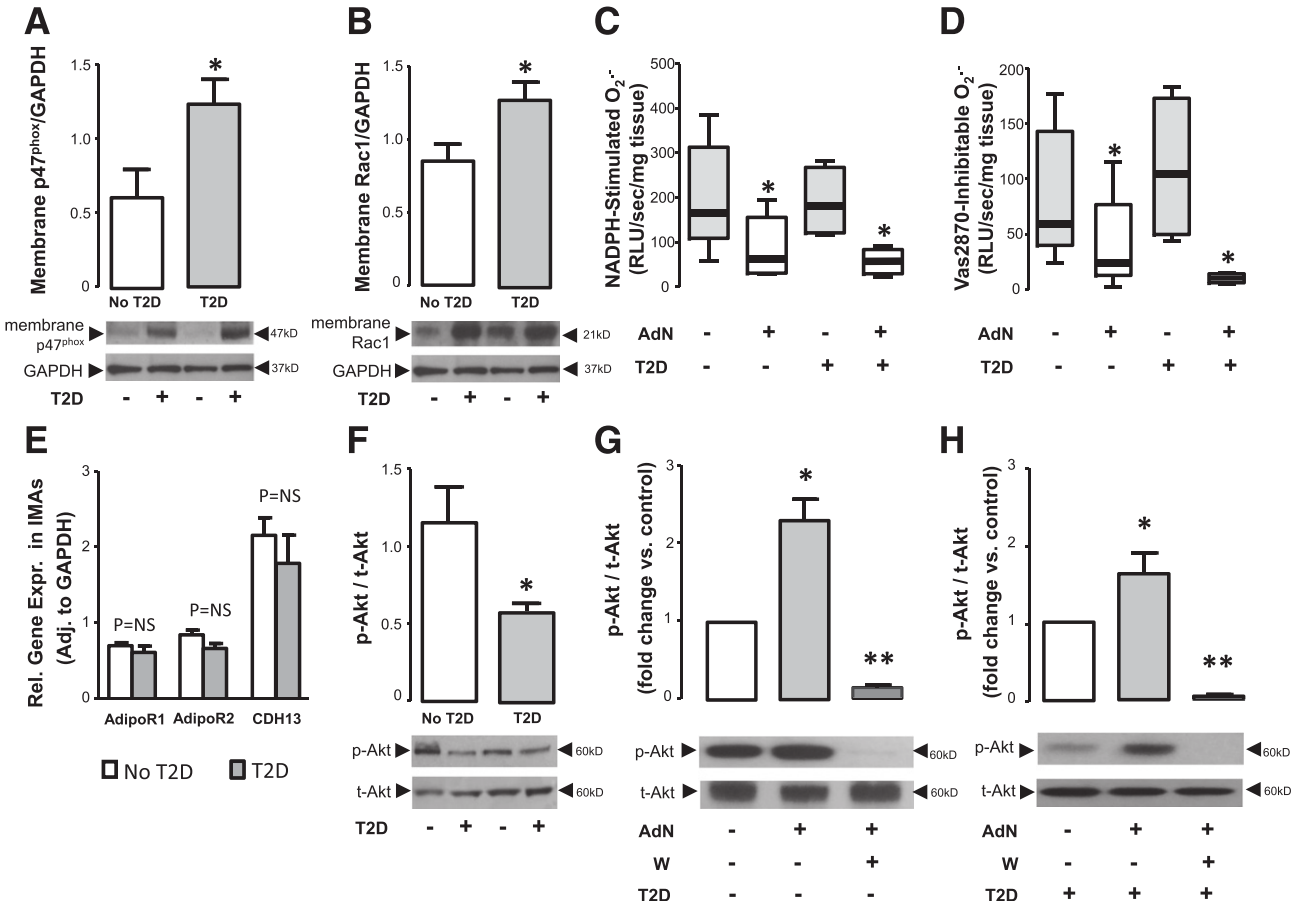

Figure 6— Membrane translocations of the $p47^{phox}$ subunit of NADPH oxidase (panel *A*) and Rac1 (panel *B*) were significantly higher in IMAs from patients with type 2 diabetes (T2D, $n = 4$) compared with patients without T2D ($n = 5$). Incubation of IMAs with adiponectin (AdN) (10 μg/mL for 6 h) led to a significant reduction in NADPH-stimulated (panel *C*) and Vas2870-inhibitable (panel *D*) $O_2^{\cdot -}$ generation, irrespective of the presence of type 2 diabetes (T2D, $n = 4$; no T2D, $n = 5$). In human IMAs, the gene expression of adiponectin receptors AdipoR1, AdipoR2, and CDH13 was not different between patients with type 2 diabetes ($n = 23$) and those without type 2 diabetes ($n = 71$) (panel *E*). The ratio of phosphorylated Akt at Ser473 (p-Akt) to total Akt (t-Akt) was significantly lower in IMAs from patients with type 2 diabetes ($n = 4$) compared with those without type 2 diabetes ($n = 5$), suggesting reduced activity of Akt in the presence of type 2 diabetes (panel *F*). Despite this, the incubation of serial IMA segments with adiponectin (100 μg/mL for 6 h) increased the p-Akt/t-Akt ratio, irrespective of the presence of type 2 diabetes, an effect abolished by the PI3K/Akt inhibitor wortmannin (W) (panels *G* and *H*) ($n = 8$). Values are presented as the mean ± SEM (panels *A*, *B*, and *E–H*) or the median (25th–75th percentiles) (panels *C* and *D*). $^*P < 0.05$ vs. no T2D (panels *A*, *B*, *E*, and *F*) or vs. control (no AdN[adiponectin]) (panels *C*, *D*, *G*, and *H*); $^{**}P < 0.01$ vs. control (panels *G* and *H*). Adj., adjustment; Expr., expression; NS, not significant; Rel., relative; RLU, relative light units; sec., seconds.

(but not of IL-6) are reduced in individuals with type 2 diabetes and IR and are also inversely correlated with NADPH oxidase activity in the human arterial wall. By using a Mendelian randomization approach, we demonstrate that the genetic variability of *ADIPOQ* leading to reduced adiponectin levels increases NADPH oxidase–derived $O_2^{\cdot -}$ levels in human arteries. We also demonstrate that adiponectin induces PI3K/Akt-mediated inhibition of Rac1 activation and membrane translocation, as well as the downregulation of $p22^{phox}$ gene expression. However, we show a paradoxical positive association between adiponectin gene expression in PVAT and NADPH oxidase–derived $O_2^{\cdot -}$ in the underlying arterial wall. To explain this phenomenon, we now demonstrate a PPAR-γ–mediated upregulation of *ADIPOQ* gene in PVAT in response to 4-HNE released from the vascular wall as a result of diabetes-related activation of NADPH oxidase. This novel interplay among vascular PI3K/Akt/NADPH oxidase, 4-HNE release, and PPAR-γ/adiponectin signaling in the human PVAT implies that PVAT is a critical regulator of the arterial redox state, protecting the human vessels from vascular oxidative stress.

Oxidative stress plays a pivotal role in the pathogenesis of vascular disease (24). The activity of vascular NADPH oxidase, a major source of $O_2^{\cdot -}$, is increased in patients with type 2 diabetes (2,25,26), a finding replicated in our cohort. Although NADPH oxidase is a major source of $O_2^{\cdot -}$ in the human body, estimating its activity directly in the human vascular wall is challenging, and any indirect estimation by measuring circulating biomarkers of oxidative stress is unreliable (27). We now demonstrate that systemic oxidative stress (characterized by plasma MDA or 4-HNE levels) is not related to NADPH oxidase activity in the human arterial wall, suggesting that local

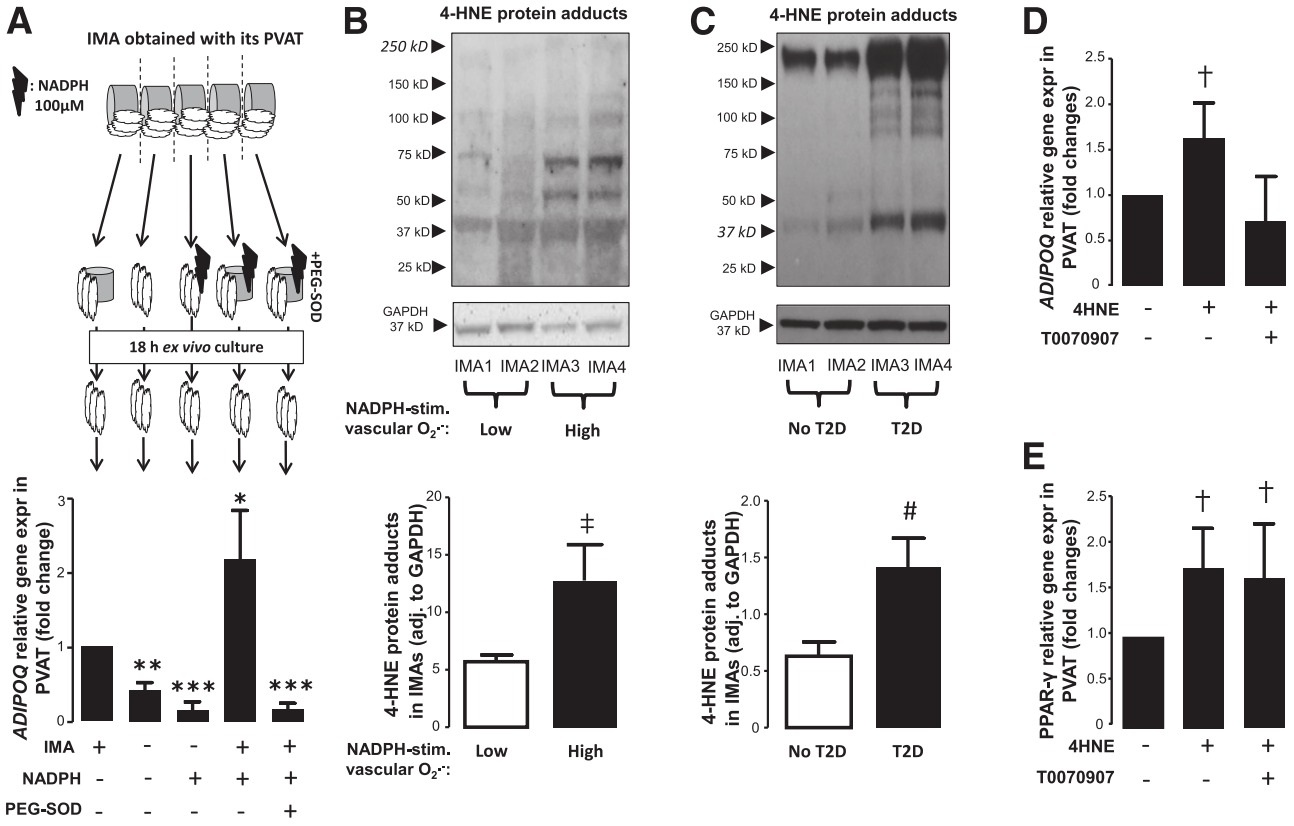

Figure 7—Separation of PVAT from its underlying IMA led to a reduction in *ADIPOQ* gene expression after 18 h of incubation; conversely, stimulation of NADPH oxidase–derived $O_2^{\cdot -}$ production by NADPH (100 μmol/L) significantly upregulated *ADIPOQ* gene expression in PVAT (panel *A*, $n = 10$). This was not through the direct effects of NADPH on PVAT, as evidenced by a downregulation of adiponectin gene expression in PVAT incubated with NADPH alone. This is further reinforced by the fact that scavenging $O_2^{\cdot -}$ radicals with PEG-SOD prevented the upregulation of *ADIPOQ* in PVAT after coincubation with the underlying IMA and NADPH (panel *A*). Western blotting for 4-HNE protein adducts revealed that IMAs with higher NADPH-stimulated $O_2^{\cdot -}$ exhibited higher levels of 4-HNE protein adducts (panel *B*) (representative blots from 4 of 9 patients). Similarly, IMAs from patients with type 2 diabetes (T2D, $n = 4$) also exhibited higher levels of 4-HNE protein adducts (panel *C*) compared with patients with no T2D ($n = 5$). Incubation of peri-IMA PVAT with 4-HNE (30 μmol/L) for 16 h upregulated *ADIPOQ* gene expression, an effect that was prevented by the PPAR-γ activity inhibitor T0070907 (10 μmol/L, $n = 11$) (panels *D* and *E*). Values are presented as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control (IMA [+] NADPH [−] PEG-SOD [−]) (panel *A*); ‡$P < 0.05$ vs. IMAs with low NADPH-stimulated $O_2^{\cdot -}$ (panel *B*); #$P < 0.05$ vs. IMAs from patients with no T2D (panel *C*); †$P < 0.05$ vs. control (4HNE [−]/T0070907 [−]) (panels *D* and *E*). adj., adjustment; expr, expression; Stim., stimulated.

mechanisms are more important in regulating the vascular redox state.

Adipose tissue produces adipokines with either proinflammatory (e.g., IL-6) or anti-inflammatory (e.g., adiponectin) potential, and the balance between these molecules is different in the various adipose tissue depots. Studies in adiponectin knockout mice (7) have shown increased NADPH oxidase activity in these animals. Given that type 2 diabetes is associated with increased activity of NADPH oxidase in experimental models (28,29) and the vascular wall (26,30), hypoadiponectinemia (a key feature in obesity and type 2 diabetes) (7) might be a link between type 2 diabetes and vascular disease pathogenesis. At a clinical level, adiponectin released by PVAT exerts vasodilatory effects on human microvessels (31), while weight loss leading to increased adiponectin levels at the same time improves endothelial function and reduces serum NOX2 levels (32). We have recently shown that adiponectin affects the vascular redox state by regulating eNOS coupling in the vascular endothelium (15), but it is unclear how a large molecule like adiponectin that is released from PVAT can reach the vascular endothelium and exert a paracrine effect. It is possible that the suppression of $O_2^{\cdot -}$ generation in the outer layers of the vascular wall could improve the overall vascular redox state, leading to secondary effects on the vascular endothelium (e.g., by affecting endothelial tetrahydrobiopterin oxidation [33] and eNOS coupling [15]). Indeed, we now demonstrate that type 2 diabetes and reduced circulating levels of adiponectin are related to increased NADPH oxidase activity in the human arterial wall, even after correcting for other cardiovascular risk factors such as smoking or hypertension. The genetic variability of the *ADIPOQ* locus, which defines the capacity of adipose tissue to release adiponectin (15,19), also leads to parallel effects on vascular NADPH oxidase activity.

We now demonstrate that *ADIPOQ* gene expression and adiponectin release from Th-AT (which is distant from large vessels) are inversely correlated with NADPH oxidase activity in the human arterial wall. However, we also present a positive correlation between *ADIPOQ* gene expression in PVAT attached to the human IMA and NADPH oxidase activity in the vessel wall, questioning the causal role of adiponectin in the regulation of the vascular redox state in humans.

To explore the hypothesis that adiponectin has a direct effect on the vascular redox state, we performed ex vivo experiments with human IMA segments and demonstrate, for the first time in humans, that adiponectin directly suppresses NADPH oxidase activity in all layers of the vascular wall (vascular smooth muscle cells and endothelium) by preventing the activation/membrane translocation of Rac1 (a subunit of NADPH oxidases, which are critical for their activation), in a PI3K/Akt-dependent way. Akt has been shown to inhibit Rac1-GTP binding through the phosphorylation of Rac1 at Ser71 (34). We now show that adiponectin increases Akt activity in the human IMA by enhancing phosphorylation at Ser473. Therefore, this adiponectin-mediated increase in Akt activity could explain the direct NADPH oxidase–suppressing effects of adiponectin. Moreover, we observe that the prolonged exposure of human arteries to adiponectin downregulates the $p22^{phox}$ gene (a critical subunit of NADPH oxidase), resulting in reduction of its protein level via a PI3K/Akt-dependent mechanism. This is compatible with recent reports (35) showing that adiponectin knockout mice display a marked upregulation of $p22^{phox}$ expression, which is prevented by supplementation with adiponectin, acting through an Akt/glycogen synthase kinase-3β/β-catenin–mediated pathway.

The presence of type 2 diabetes has been linked to adiponectin "resistance" in peripheral tissues (adipose tissue and skeletal muscle) due to reduced expression of adiponectin receptors (36). In our study, we observe that the ability of adiponectin to suppress NADPH oxidase activity in human arteries is preserved in patients with type 2 diabetes, and the expression of adiponectin receptors (AdipoR1, AdipoR2 and CDH13) is similar between patients with and without type 2 diabetes. Similarly, while vascular Akt activity is reduced in patients with type 2 diabetes, exogenously administered adiponectin is able to activate Akt in the IMA wall. Therefore, we conclude that the increased Akt-mediated NADPH oxidase activity observed in the human arterial wall in type 2 diabetes is mainly due to reduced adiponectin levels rather than to the impaired responsiveness of these vessels to adiponectin.

In this study, we also demonstrate that an increased level of NADPH oxidase–derived $O_2^{\cdot -}$ in the arterial wall is correlated with increased *ADIPOQ* gene expression in the PVAT surrounding it. By using an ex vivo model of human IMA and PVAT cocultures, we show for the first time that the activation of NADPH oxidase in the human arterial wall leads to the local production of oxidation products (e.g., 4-HNE), which are able to upregulate PPAR-γ–mediated *ADIPOQ* expression in the neighboring PVAT. These findings indicate that oxidation products released from the arterial wall may represent "rescue signals" toward PVAT to increase the expression of adiponectin as a local control mechanism of vascular NADPH oxidase activity.

In conclusion, this is the first study demonstrating the role of adiponectin in the direct regulation of NADPH oxidase activity in the human arterial wall, suggesting that hypoadiponectinemia is a key feature in the development of the vascular complications of type 2 diabetes. In addition, we demonstrate for the first time in humans that adiponectin produced in PVAT may exert a paracrine effect on the underlying arterial wall by suppressing NADPH oxidase activity via a PI3K/Akt-mediated deactivation of Rac1 and the downregulation of $p22^{phox}$ gene expression. Importantly, the activation of vascular NADPH oxidase and the presence of type 2 diabetes lead to local production of oxidation products from the human IMA (e.g., 4-HNE), which are able to upregulate adiponectin gene expression in PVAT. This cross-talk between PI3K/Akt/NADPH oxidase in the human arterial wall and PPAR-γ/*ADIPOQ* signaling in the surrounding PVAT is now identified as a rational therapeutic target to prevent the vascular complications of type 2 diabetes.

Funding. This work was supported by a Research Fellowship from the Heart Failure Association of the European Society of Cardiology to A.S.A. and by grants from the British Heart Foundation Centre of Research Excellence-Oxford (RE/08/004 to M.M. and C.A.) and the British Heart Foundation (FS/11/66/28855 and PG/13/56/30383 to C.A.).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. A.S.A. participated in patient recruitment and collected samples, and helped to perform the experiments and review the manuscript. M.M. helped to perform the experiments and analyze the data, and contributed to the writing of the manuscript. P.C., C.S., C.P., F.S., R.L., and C.B. helped to perform the experiments, analyze the data, and review the manuscript. L.H. participated in patient recruitment. R.D.S., M.P., R.S., and G.K. provided surgical samples and helped to review the manuscript. J.D. and S.R. provided advice, expertise, and reagents, and helped to review the manuscript. D.T., B.C., and K.M.C. provided advice and discussed and reviewed the manuscript. B.K. helped to perform the experiments and analyze the data, provided advice, and discussed and reviewed the manuscript. C.A. developed the hypothesis, designed the experiments, coordinated and directed the project, participated in data analysis, and contributed to the writing of the manuscript. C.A. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 2013 American Heart Association Scientific Sessions, Dallas, TX, 16–20 November 2013.

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