Adiponectin As A Link Between Type 2 Diabetes Mellitus And Vascular NADPH-Oxidase Activity In The Human Arterial Wall: The Regulatory Role Of Perivascular Adipose Tissue

Alexios S Antonopoulos¹*, Marios Margaritis¹*, Patricia Coutinho¹, Cheerag Shirodaria¹,

Constantinos Psarros³, Laura Herdman¹, Fabio Sanna¹, Ravi De Silva², Mario Petrou²,

Rana Sayeed², George Krasopoulos², Regent Lee¹, Janet Digby¹, Svetlana Reilly¹,

Constantinos Bakogiannis³, Dimitris Tousoulis³, Benedikt Kessler⁴, Barbara Casadei¹,

Keith M Channon¹, Charalambos Antoniades¹

¹: Cardiovascular Medicine Division, University of Oxford, UK

²: Department of Cardiac Surgery, John Radcliffe Hospital, Oxford UK

³: 1st Department of Cardiology, Athens University Medical School, Greece

⁴: Nuffield Department of Medicine, University of Oxford

Running title: “PVAT and arterial NADPH-oxidase in humans”

Word count: Text 4848, Figures 7 (6 greyscale, 1 color), Suppl. Figures 2, References: 36

*Authors contributed equally to the study

Corresponding author:
Charalambos Antoniades MD PhD,
Associate Professor of Cardiovascular Medicine,
Cardiovascular Medicine Division, University of Oxford
John Radcliffe Hospital West Wing Level 6, Headley Way, Oxford OX3 9DU, UK
Tel: +44-1865-221870, Fax: +44-1865-740352, e-mail: antoniad@well.ox.ac.uk
Abstract

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 (IMA) with their perivascular adipose tissue (PVAT) and thoracic adipose tissue (Th-AT) 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 O$_2^\cdot$.

Genetic variability of ADIPOQ and circulating adiponectin (but not IL-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 down-regulating p22$^{phox}$, through a PI3K/Akt-mediated mechanism. In ex vivo co-incubation models of IMA/PVAT, activation of arterial NADPH-oxidase triggered a PPAR-$\gamma$ mediated up-regulation of adiponectin gene in the neighboring PVAT, via the release of vascular oxidation products. We demonstrate for the first time in humans that reduced adiponectin in type 2 diabetes stimulates vascular NADPH-oxidase, while PVAT “senses” increased NADPH-oxidase activity in the underlying vessel and responds by up-regulating adiponectin gene expression. This PVAT-vessel interaction is identified as a novel therapeutic target for the prevention of vascular complications of type 2 diabetes.

**Key words**: Adiponectin; Superoxide; NADPH oxidase; Cardiovascular disease(s); Adipose Tissue; Atherogenesis
NADPH-oxidase, a potent source of superoxide anions (O$_2^-$) in the vascular wall (1), is directly implicated in atherogenesis (2-4). The presence of type 2 diabetes mellitus 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 type 2 diabetes are unclear.

Adipose tissue releases both pro-inflammatory (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 type 2 diabetes and obesity (7). While some studies support a causal link between low adiponectin and impaired glucose tolerance (9), others have failed to find such evidence (10). Low circulating adiponectin is associated with increased cardiovascular risk in healthy individuals (11), although recent studies suggest that in advanced cardiovascular disease states circulating adiponectin is increased as a “stress hormone”, and its levels become predictive of adverse clinical outcome (12). On the other hand, pro-inflammatory cytokines released from the human adipose tissue have well established pro-atherogenic potential, and they also predict adverse clinical outcome in advanced cardiovascular disease states (13).

The balance between pro- 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 pro-inflammatory 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 anti-contractile effects on the underlying vessels (14), while it 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 type 2 diabetes mellitus (16).

In the present study we define the role of type 2 diabetes in the regulation of vascular redox state, focused 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 we explore the cross-talk between vascular NADPH-oxidase and PPAR-γ signalling controlling adiponectin expression in the human PVAT.

**Methods**

**Population and Protocol**

The population of Study 1 consisted of 386 patients (Table 1) undergoing elective coronary artery bypass grafting surgery (CABG). Exclusion criteria were any inflammatory, infectious, liver or renal disease or malignancy. Patients with heart failure or those receiving non-steroidal anti-inflammatory drugs, dietary supplements or antioxidant vitamins were also excluded. Blood samples were obtained on the morning of the surgery. During CABG, internal mammary artery (IMA) segments were harvested by preserving their PVAT. In addition to the PVAT surrounding the IMA, samples of thoracic fat (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 4h, to quantify the release of adiponectin and interleukin-6 (IL-6). In study 2, we included 67 additional patients undergoing CABG (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.

**Measurements of Circulating Biomarkers**

Serum total adiponectin, IL-6 and high molecular weight (HMW) adiponectin were measured by enzyme linked immunosorbent assays (ELISA) (BioVendor, Brno, Czech Republic, R&D systems USA and Otsuka pharmaceutical Co respectively). Plasma malonyldialdehyde (MDA), a marker of systemic oxidative stress, was quantified by using the thiobarbituric acid reactive substances (TBARS) 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 was measured by Chemiluminescent Microparticle Immunoassay and serum glucose by the hexokinase method using commercial kits (ABBOTT, Wiesbaden Germany). HOMA-IR was calculated by using the formula (glucose x insulin)/405, with glucose measured in mg/dL and insulin in mU/L (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 SNPs have a known effect adiponectin levels in recent genome wide association studies (19).
Vascular Superoxide 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 positive control. Previous observations from our group have yielded a very strong correlation between NADPH-stimulated O$_2^\cdot$ in intact and homogenised 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 hours. The secretion of total adiponectin, IL-6 and HMW adiponectin in adipose tissue culture supernatants was measured by ELISA (BioVendor, Brno, Czech Republic; R&D systems USA and Otsuka pharmaceutical Co respectively).

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-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 qRT-PCR (TaqMan Probes (Life Technologies)), using PPIA (cyclophilin) or GAPDH as housekeeping gene respectively.
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 following the same exclusion criteria as for Study 1. Briefly, serial rings from the same vessel were incubated in oxygenated (95%O\textsubscript{2}/5%CO\textsubscript{2}) Krebs-HEPES Buffer in the presence or absence of recombinant full-length adiponectin 10 µg/ml (BioVendor, RD172029100) for 6 or 18 hours as stated. The effect of adiponectin on NADPH-stimulated O\textsubscript{2}·− was quantified by lucigenin chemiluminesence (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 PI3 kinase/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\textsubscript{2}·− production was determined in vessel cryosections with oxidative fluorescent dye dihydroethidium (DHE) as previously described (20). Serial IMA rings were incubated ±adiponectin 10 µg/ml for 6 hours. 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 & p47\textsuperscript{phox}

Rac1 activation was evaluated by a commercially available affinity precipitation assay using the PAK1-PBD conjugated glutathione agarose beads (Millipore, Temecula, USA)(6). To
estimate membrane translocation of Rac1 & p47phox, we performed differential centrifugation for isolation of membrane proteins, and membrane-translocated Rac-1 or p47phox protein was determined by Western immunobloting as previously described (6).

**Western Blots**

Western immunoblotting was used to examine the direct effects of adiponectin on NOX1, NOX2, NOX4 (antibodies by Abcam, Cambridge, UK), p47phox, p67phox, phospho-Akt (Ser473), pan-Akt (Cell Signaling, 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/without type 2 diabetes, exhibiting high/low NADPH-stimulated O$_2^-$ generation.

**Total Antioxidant Capacity of Human IMAs**

To investigate differences in the total antioxidant capacity of IMAs from patients with/without type 2 diabetes, we used a commercially available kit (Cell Biolabs, San Diego, CA). This kit quantifies Cu$^{2+}$-reducing equivalent/mg protein as an index of the overall antioxidant capacity of the tissue.

**Co-Cultures 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 co-incubations of human IMA with/without their respective PVAT. Briefly, PVAT was incubated in 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 + 100 μmol/l NADPH (to stimulate NADPH-oxidase) or with its IMA tissue + 100 μmol/l
NADPH + 300 U/ml 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 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 SD 0.38, $\alpha=0.05$ and power 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 $\alpha=0.05$, power 90% and SD for a difference in the response of the pairs 0.2.

In the clinical studies, continuous variables between 3 groups were compared by using one-way ANOVA, while comparisons between 2 groups were performed by unpaired t-tests, corrected by using Bonferroni post-hoc correction for multiple testing when more than 1 comparisons were performed. Categorical variables were compared by using chi-square test, as appropriate. Correlations between continuous variables were assessed by calculating
Pearson’s or Spearman’s 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 repeated measures ANOVA followed by paired samples t-test for individual comparisons with Bonferroni correction when multiple tests, as appropriate. Wilcoxon paired-rank test followed by Bonferroni correction was used to compare values expressed as fold-changes vs control.

Linear regression was performed by using log(NADPH-stimulated O$_2^\cdot$) as dependent variable. As independent variables we used log(serum adiponectin) or the ADIPOQ genotype plus those of the clinical demographic characteristics (age, gender, type 2 diabetes, smoking, dyslipidemia, 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 threshold to remove a variable from the model. All statistical tests were performed by using SPSS v20.0 and P<0.05 was considered statistically significant.

**Results**

The patients’ demographic characteristics are presented in Table 1. We first demonstrate that insulin resistance (IR), as defined by HOMA-IR $\geq$2.8, and type 2 diabetes are both associated with reduced circulating adiponectin in our cohort (Figure 1A). Similarly, IR/type 2 diabetes were linked to increased NADPH oxidase-derived O$_2^\cdot$ in the human IMA (Figure 1B). There was no significant association between IR or type 2 diabetes and circulating MDA (Figure 1C) or 4-HNE (Figure 1D, both markers of systemic oxidative stress), suggesting that the impact of type 2 diabetes and IR on vascular redox state can not be accurately monitored by circulating biomarkers of oxidative stress. There was no significant correlation between plasma MDA and NADPH-oxidase-derived O$_2^\cdot$ 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 was related to NADPH-oxidase activity in these vessels and found that NADPH-stimulated O$_2^\cdot$ was lower in patients with high circulating adiponectin (Figure 2A). In contrast, serum IL-6 was not related to IMA’s NADPH-stimulated O$_2^\cdot$ (Figure 2B). These results imply that the increased NADPH-oxidase activity observed in IR/type 2 diabetes could be caused, at least in part, by reduced circulating adiponectin. To explore this concept, we then tested whether genetic variability of ADIPOQ gene encoding adiponectin (rs17366568 and rs266717 SNPs) (21; 22) affects NADPH-oxidase activity in the IMA, in an attempt to establish the direction of the observed association between IR/type 2 diabetes, adiponectin levels and arterial NADPH-oxidase activity. 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 adiponectin (Figure 2C) and adiponectin gene expression in Th-AT (Figure 2D) but not PVAT (P=NS, data not shown). Importantly, the number of rs17366568G and rs266717T alleles was inversely related with the presence of type 2 diabetes (Spearman rho= -0.176, P=0.001). The number of rs17366568G and rs266717T alleles was also negatively related with NADPH-stimulated O$_2^\cdot$ in IMA segments (Figure 2E), while it had no significant impact on systemic oxidative stress, as estimated by plasma MDA (Figure 2F) or systemic inflammation (defined by serum IL-6, data not shown). In univariate analysis, log(NADPH-stimulated O$_2^\cdot$) was related with both log(serum adiponectin) (r=-0.219, P<0.0001) and type 2 diabetes (rho=0.195, P=0.001), but also with smoking status (rho=0.172, P=0.003) and hypertension (rho=0.149, P=0.009). To correct the association between adiponectin/type 2 diabetes and NADPH-oxidase activity for the confounding effects of smoking and hypertension, we performed multivariable analysis in which we confirmed that in human arteries, log(NADPH-stimulated O$_2^\cdot$) is related with log(serum adiponectin) (β(SE): -0.233(0.074), P=0.002) and type 2 diabetes.
and smoking (β(SE):0.088(0.031), P=0.004), with $R^2$ for the model 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 $O_2^-$) (β(SE):-0.081(0.025), P=0.001) independently of hypertension: (β(SE):0.150(0.053), P=0.005) and smoking: (β(SE):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 adiponectin (but not IL-6) might be a regulator of NADPH-oxidase activity in the human arterial wall, inter-related with diabetes in a way that it 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 correlates with NADPH-oxidase activity in the human arteries. We observed that both gene expression (Figure 3A) and release of total (Figure 3B) or HMW (Supplementary Figure S1) adiponectin from Th-AT were inversely correlated with NADPH-stimulated $O_2^-$ in these vessels. Paradoxically, ADIPOQ gene expression from PVAT was positively correlated with NADPH oxidase-derived $O_2^-$ in the underlying arterial wall (Figure 3C), 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 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 $O_2^-$ in the human arteries (Figures 3D-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 and vascular oxidative stress in humans, we performed additional
mechanistic *ex vivo* experiments with serial IMA segments obtained from 67 patients undergoing CABG. In these experiments, incubation of these arterial segments with adiponectin 10 µg/ml, a biologically relevant concentration (11), for 6 hours significantly reduced $O_2^-$ and its Vas2870-inhibitable $O_2^-$ fraction in all layers of the vascular wall (Figure 4). Similarly, the NADPH-stimulated $O_2^-$ (Figure 5A) and its Vas2870-inhibitable fraction (Figure 5B) were significantly reduced by adiponectin, an effect reversed by the PI3k/Akt signalling 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 incubation of IMA segments with adiponectin 10 µg/ml for 6 hours 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 hours, which was prevented by wortmannin (Figure 5C). This effect was not translated into a reduction in vascular p22$^{phox}$ protein levels at that early time-point (Figure 5D), 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 activation of Rac1, as evidenced by a reduction in GTP-bound Rac1, (Figure 5E), and its translocation to the membrane (Figure 5F), 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 hours of incubation of human IMAs with adiponectin, we performed longer-term incubations (for 18h), where we confirmed that the effect of adiponectin on vascular $O_2^-$ in these vessels (Figures 5G and H) was now also
accompanied by a reduction in the protein level of vascular p22phox subunit (Figure 5I),
documenting a second sub-acute effect of adiponectin on NADPH-oxidase.

To explore the role of adiponectin in vascular NADPH-oxidase regulation in type 2
diabetes, we performed additional experiments in IMAs obtained from patients with/without
type 2 diabetes. We observed that type 2 diabetes is related with increased membrane
translocation of the cytosolic subunits p47phox and Rac1 of NADPH-oxidase, explaining the
activation of vascular NADPH-oxidase in these patients (Figure 6A and 6B). Interestingly,
we observed that adiponectin (10 µg/ml for 6h) reduced NADPH oxidase-derived O$_2$$^-$ in
IMAs from patients either with or without type 2 diabetes (Figure 6C and 6D). 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/without
type 2 diabetes (Figure 6E). We also observed lower Akt phosphorylation at Ser473 (an
activation site) in IMAs obtained from patients with type 2 diabetes compared to those
without diabetes (Figure 6E), and incubation of these vessels with adiponectin induced
phosphorylation at Ser473/activation of Akt irrespective of the presence of type 2 diabetes
(Figure 6G and 6H).

To explain the positive association between NADPH-stimulated O$_2$$^-$ in IMA and ADIPOQ
gene expression in the surrounding PVAT, we hypothesised that vascular oxidative stress
may trigger the expression of adiponectin gene in PVAT. To test this hypothesis, we
performed co-incubation experiments of PVAT with/without its respective IMA for 18 h, as
well as after stimulation of vascular NADPH-oxidase by using NADPH (100 µmol/L)
(Figure 7A). We found that removal of the underlying vascular tissue resulted into a ~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 direct
effects of NADPH on PVAT (e.g. via effects on P2X receptors (23)), because incubation of
PVAT + NADPH without the underlying vessel still led to a reduction in \textit{ADIPOQ} gene expression (Figure 7A). Importantly, scavenging reactive oxygen species with PEG-SOD not only prevented the NADPH-induced up-regulation of \textit{ADIPOQ} gene, but it also resulted in its downregulation below the baseline (control) expression levels (PVAT+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 \textit{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 \textit{ADIPOQ} gene expression via changes in vascular PPAR-\(\gamma\) signalling in the neighbouring PVAT. Increased levels of 4-HNE protein adducts were observed in human IMAs with higher NADPH-oxidase activity, confirming that NADPH-oxidase-derived \(O_2^-\) leads to the production of 4-HNE in these vessels (Figure 7B). Similarly, IMAs obtained from patients with type 2 diabetes had higher levels of these adducts (4-HNE- and MDA-protein adducts) compared to patients without diabetes (Figure 7C & Supplementary Figure S2), confirming the increased vascular oxidative stress in type 2 diabetes. In addition, IMAs from patients with type 2 diabetes also had significantly reduced total antioxidant capacity (Supplementary Figure S2), suggesting depletion of endogenous vascular antioxidant systems in 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 up-regulation of \textit{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) up-regulated \textit{ADIPOQ} gene expression (by 63\% vs control, Figure 7D and Supplementary Figure S2). 4HNE also upregulated PPAR-\(\gamma\) gene (Figure 7E), while it had no
effect on the expression of *IL-6* gene in the human PVAT (reduced by 10% versus 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 (Figures 7D and 7E).

**Discussion**

In the present study we examine the role of adiponectin as a link between type 2 diabetes and vascular redox state. We demonstrate that circulating adiponectin (but not IL-6) is reduced in type 2 diabetes and IR and is 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$_2^.-$ in human arteries. We also demonstrate that adiponectin induces PI3K/Akt-mediated inhibition of Rac1 activation and membrane translocation, as well as down-regulation of p22phox gene expression. However, we show a paradoxical positive association between adiponectin gene expression in PVAT and NADPH-oxidase-derived O$_2^.-$ in the underlying arterial wall. To explain this phenomenon, we now demonstrate a PPAR-γ-mediated up-regulation 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 between vascular PI3K/Akt/NADPH-oxidase, 4-HNE release and PPAR-γ/adiponectin signalling in the human PVAT implies that PVAT is a critical regulator of arterial redox state, protecting the human vessels from vascular oxidative stress.

Oxidative stress plays a pivotal role in vascular disease pathogenesis (24). The activity of vascular NADPH-oxidase, a major source of O$_2^.-$, 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$_2^.-$ 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 (characterised by plasma MDA or 4-HNE) is not related to NADPH-oxidase activity in the human arterial wall, suggesting that local mechanisms are more important in regulating vascular redox state.

Adipose tissue produces adipokines with either pro-inflammatory (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 on 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), hypoadiponectinaemia (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 vascular redox state by regulating eNOS coupling in the vascular endothelium (15), but it is unclear how a large molecule like adiponectin released from PVAT can reach the vascular endothelium and exert a paracrine effect. It is possible that 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 adiponectin are related with 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 ADIPOQ locus that 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 to 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 vascular redox state in humans.

To explore the hypothesis that adiponectin has a direct effect on 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 (subunit of NADPH oxidases, critical for their activation), in a PI3 kinase/Akt-dependent way. Akt has been shown to inhibit Rac1-GTP binding through 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 prolonged exposure of human arteries to adiponectin down-regulates p22\textsuperscript{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 showing that adiponectin knock-out mice display a marked upregulation of p22\textsuperscript{phox} expression, which is prevented by supplementation with adiponectin, acting through an Akt/GSK3β/β-catenin-mediated pathway (35).

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 type 2 diabetes, and the expression of
adiponectin receptors (AdipoR1, AdipoR2 and CDH13) is similar between patients with vs those without type 2 diabetes. Similarly, while vascular Akt activity is reduced in 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 impaired responsiveness of these vessels to adiponectin.

In this study we also demonstrate that increased NADPH-oxidase-derived $\text{O}_2^\cdot$ in the arterial wall is correlated with increased $\text{ADIPOQ}$ gene expression in PVAT surrounding it. By using an ex vivo model of human IMA and PVAT co-cultures, we show for the first time that activation of NADPH-oxidase in the the human arterial wall leads to the local production of oxidation products (e.g. 4-HNE) which are able to up-regulate PPARγ-mediated $\text{ADIPOQ}$ expression in the neighbouring PVAT. These findings indicate that oxidation products released from the arterial wall may represent “rescue signals” towards 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 regulation of NADPH-oxidase activity directly in the human arterial wall, suggesting that hypo-adiponectinemia 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 supressing NADPH-oxidase activity via a PI3K/Akt/-mediated deactivation of Rac1 and down-regulation of p22$^{\text{phox}}$ gene expression. Importantly, activation of vascular NADPH-oxidase and presence of type 2 diabetes lead to local production of oxidation products from the human IMA (such as 4-HNE) which are able to up-regulate adiponectin gene expression in PVAT. This cross-talk between PI3K/Akt/NADPH-oxidase in the human arterial wall and
PPAR-γ/ADIPOQ signalling in the surrounding PVAT is now identified as a rational therapeutic target to prevent the vascular complications of type 2 diabetes.

Article Information

Funding. This work was supported by a Research Fellowship from the Heart Failure Association of the European Society of Cardiology to A.A. and grants from the British Heart Foundation (FS/11/66/28855 and PG/13/56/30383 to C.A.) and the British Heart Foundation Centre of Research Excellence - Oxford (RE/08/004 to M.M./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, samples collection, performed experiments, and reviewed the manuscript. M.M. performed experiments, analyzed the data and contributed to the writing of the manuscript. P.C., B.K., C.S., C.P., R.L., C.B. and F.S. performed experiments, analyzed the data, and reviewed the manuscript, L.H. participated in patient recruitment. R.D.S., M.P., R.S. and G.K. provided surgical samples and reviewed the manuscript. J.D. and S.R. provided advice, expertise and reagents, and reviewed the manuscript. D.T., B.C. B.K. and K.M.C. provided advice, discussed and reviewed the manuscript. C.A. developed the hypothesis, designed the experiments, coordinated and directed the project, participated in data analysis and wrote 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 Figures 2, 3 & 5 were presented as an oral presentation at the American Heart Association Scientific Sessions in Dallas, Texas, 16-20 November 2013.
References

1. Morawietz H: Endothelial NADPH oxidases: friends or foes? Basic Res Cardiol 2011;106:521-525


Table 1: Demographic characteristics of study participants

<table>
<thead>
<tr>
<th></th>
<th>Clinical Studies (Study 1)</th>
<th>Ex vivo Studies (Study 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants (n)</td>
<td>386</td>
<td>67</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65.8±0.5</td>
<td>65.5±1.3</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>83.7</td>
<td>97.0</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>68.9</td>
<td>76.1</td>
</tr>
<tr>
<td>Hyperlipidaemia (%)</td>
<td>62.4</td>
<td>77.6</td>
</tr>
<tr>
<td>Type 2 diabetes (%)</td>
<td>32.9</td>
<td>26.9</td>
</tr>
<tr>
<td>Smoking (active/ex) (%)</td>
<td>22.8/46.6</td>
<td>4.4/59.7</td>
</tr>
<tr>
<td>Body mass index (Kg/m²)</td>
<td>27.7±0.2</td>
<td>29.4±0.5</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>177.3±2.4</td>
<td>146.9±12.4</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>125.4±3.1</td>
<td>97.2±5.4</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>15.49±1.43</td>
<td>13.98±2.01</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>5.39±0.54</td>
<td>3.36±0.03</td>
</tr>
<tr>
<td>High density lipoprotein (mg/dl)</td>
<td>38.8±0.6</td>
<td>42.5±7.7</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>141.4±4.9</td>
<td>149.7±40.7</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.3±0.1</td>
<td>13.7±0.2</td>
</tr>
<tr>
<td>White blood cell count (cells/µL)</td>
<td>7288±151</td>
<td>8553±749</td>
</tr>
<tr>
<td>Platelets count (cells/µL)</td>
<td>239782±4224</td>
<td>225900±8100</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.07±0.02</td>
<td>0.94±0.04</td>
</tr>
<tr>
<td>rs266717 (CC/CT/TT) (%)</td>
<td>23.2/48.1/28.7</td>
<td>-</td>
</tr>
<tr>
<td>rs17366568 (AA/AG/GG) (%)</td>
<td>1.3/18.5/80.2</td>
<td>-</td>
</tr>
</tbody>
</table>

**Medication (%)**

- ACEi: 58.5, 46.2
- ARBs: 9.8, 16.4
- Beta blockers: 75.3, 88.0
- Aspirin/Clopidogrel: 82.1, 93.6
- Statins: 78.2, 88.0
- CCBs: 24.9, 41.7

ACEi: Angiotensin converting enzyme inhibitors; ARBs: Angiotensin receptor blockers; CCBs: Calcium channel blockers. HOMA-IR: Homeostatic model of insulin resistance
Legend to the Figures

**Figure 1.** Patients with insulin resistance (IR) or type 2 diabetes mellitus (T2DM) had significantly lower circulating levels of adiponectin (AdN, Panel A), as well as increased NADPH-oxidase-derived superoxide (O$_2^-$) in the vascular wall of the internal mammary artery (IMA) (Panel B). There was no significant association between IR/T2DM and plasma malonyldialdehyde (MDA) (Panel C) or 4-hydroxynonenal (4-HNE) levels (Panel D), both markers of systemic oxidative stress. Values are expressed as median [25$^{\text{th}}$-75$^{\text{th}}$ percentile].

**Figure 2.** Patients with high serum adiponectin (AdN) had significantly lower NADPH-stimulated superoxide (O$_2^-$) generation in the vascular wall of their left internal mammary artery (Panel A), while serum interleukin 6 (IL-6) levels were unrelated with NADPH-stimulated O$_2^-$ 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 adiponectin (Panel C) and its release from thoracic adipose tissue (Th-AT) after 4 hours of tissue culture (Panel D). By using this genetic model we observed that the number of rs17366568G/rs266717T alleles was inversely related with NADPH-stimulated O$_2^-$ in the arterial wall (Panel E). However, the genetic variability of ADIPOQ gene had no effect on plasma malonyldialdehyde (MDA) levels, a marker of systemic lipid peroxidation (Panel F). Values are expressed as median [25$^{\text{th}}$-75$^{\text{th}}$ percentile].

**Figure 3.** There was an inverse association between NADPH-stimulated O$_2^-$ in human internal mammary arteries and adiponectin gene expression (ADIPOQ gene, Panel A) and release (after 4h of tissue culture, Panel B) in thoracic adipose tissue (Th-AT). However, there was a positive association between vascular NADPH-stimulated O$_2^-$ and ADIPOQ gene expression in perivascular adipose tissue (PVAT) surrounding these vessels (Panel C). Importantly, there was no correlation between NADPH-stimulated O$_2^-$ in the human arterial
wall and interleukin-6 (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 median [25th-75th percentile].

**Figure 4.** *Ex-vivo* incubation of serial human left internal mammary artery (IMA) segments with adiponectin (AdN) 10 µg/ml for 6 h resulted into significant reduction of superoxide (O$_2^-$, red dots) production in both the vascular endothelium (white arrow heads) and the wall (yellow arrow heads, see Panels A and B). Inhibition with Vas2870 (a specific inhibitor of NADPH-oxidase) significantly suppressed O$_2^-$ production in both the endothelium and the vascular wall (Panels C and D) suggesting that adiponectin suppressed NADPH-oxidase derived O$_2^-$ signal throughout the entire vascular wall. Images of dihydroethidium staining viewed by a Zeiss LSM 510 META laser scanning confocal microscope at 40x magnification.

**Figure 5.** *Ex-vivo* incubation of serial human left internal mammary (IMA) rings (n=10) with adiponectin (AdN) 10µg/ml for 6 hours significantly reduced NADPH-stimulated O$_2^-$ (Panel A) and its Vas2870-inhibitable fraction (Panel B) as measured by lucigenin-enhanced chemiluminescence in fresh tissue samples. This effect was reversed by co-incubation with wortmannin (W, 100 nmol/L), an inhibitor of PI3-kinase/Akt pathway (Panels A and B). Incubation of human IMA segments with adiponectin for 6 hours (n=8) resulted into significant downregulation of 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 reversed by wortmannin (n=7). The effect of adiponectin (AdN) 10µg/ml incubation on NADPH-stimulated superoxide (O$_2^-$, Panel G) and its Vas2870-inhibitable fraction (Panel H) remained significant after 18h 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 medians [25\textsuperscript{th}-75\textsuperscript{th} percentiles] (Panels A, B, G and H) or means±SEM (Panels C-F and I). *P<0.05 vs control.

**Figure 6**. Membrane translocation of p47\textsuperscript{phox} subunit of NADPH-oxidase (Panel A) and Rac1 (Panel B) were significantly higher in internal mammary arteries (IMAs) from patients with type 2 diabetes (T2DM, n=4) compared to patients without T2DM (n=5). Incubation of IMAs with adiponectin (10 µg/ml for 6 hours) led to a significant reduction in NADPH-stimulated (Panel C) and Vas2870-inhibitable (Panel D) superoxide (O\textsubscript{2}\textsuperscript{·}) generation, irrespective of presence of T2DM (n=4 T2DM and n=5 no T2DM). In human IMAs, the gene expression of adiponectin receptors AdipoR1, AdipoR2 and CDH13 was not different between patients with T2DM (n=23) compared to those with no T2DM (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 T2DM (n=4) compared to those with no T2DM (n=5), suggesting reduced activity of Akt in the presence of T2DM (Panel F). Despite this, incubation of serial IMA segments with adiponectin (AdN, 100µg/ml for 6h) increased p-Akt/t-Akt ratio, irrespective of the presence of type 2 diabetes, an effect abolished by the PI3K/Akt inhibitor wortmannin (Panels G and H) (n=8). Values are presented as means±SEM (Panels A,B, E-H) or medians [25\textsuperscript{th}-75\textsuperscript{th} percentiles] (Panels C and D). *P<0.05 vs no T2DM (Panels A, B, E, and F) or vs control (no AdN, Panels C, D, G and H).

**Figure 7**. Separation of perivascular adipose tissue (PVAT) from its underlying internal mammary artery (IMA) led to a reduction in ADIPOQ gene expression after 18 h incubation; conversely, stimulation of NADPH-oxidase–derived O\textsubscript{2}\textsuperscript{·} production by NADPH (100 µmol/L) significantly up-regulated ADIPOQ gene expression in PVAT (Panel A, n=10). This was not through direct effects of NADPH on PVAT, as evidenced by a down-regulation of adiponectin gene expression in PVAT incubated with NADPH alone. This is further
reinforced by the fact that scavenging \(O_2^-\) radicals with PEG-SOD prevented the up-regulation of \(ADIPOQ\) in PVAT after co-incubation with the underlying IMA and NADPH (Panel A). Western blotting for 4-HNE protein adducts revealed that IMAs with higher NADPH-stimulated \(O_2^-\) exhibited higher levels of 4-hydroxy-nonenal (4-HNE) protein adducts (Panel B, representative blots from 4 patients out of n=9). Similarly, IMAs from patients with type 2 diabetes (T2DM, n=4) also exhibited higher levels of 4-HNE protein adducts (Panel C) compared to patients with no T2DM (n=5). Incubation of peri-IMA PVAT with 4-HNE (30 µmol/L) for 16 h up-regulated \(ADIPOQ\) gene expression, an effect that was prevented by the PPAR-\(\gamma\) activity inhibitor T0070907 (10 µmol/L, n=11, Panels D and E).

Values are presented as means±SEM. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\) vs control (IMA (+) NADPH (-) PEG-SOD (-) for Panel A); ‡ \(P<0.05\) vs. IMAs with low NADPH-stimulated \(O_2^-\) (Panel B); # \(P<0.05\) vs. IMAs from patients with no T2DM (Panel C); † \(P<0.05\) vs. control (4HNE (-) / T0070907 (-), Panels D&E).
Figure 1

(A) Serum AdN (μg/ml)

(B) NADPH-stimulated O₂⁻ (RLU/sec/mg tissue)

(C) Plasma MDA (μmol/L)

(D) Plasma 4HNE (umol/L)

NG, IR, T2DM
Figure 3

(A) NADPH-stimulated \(O_2^\cdot\) (RLU/sec/mg tissue).

(B) Tertiles ADIPOQ expr. in Th-AT

(C) Tertiles AdN release from Th-AT

(D) Tertiles IL6 expr. in Th-AT

(E) Tertiles IL6 release from Th-AT

(F) Tertiles IL6 expr. in PVAT

P<0.05

P=NS
Figure 5

(A) NADPH-Stimulated \( \text{O}_2^\cdot \) (RLU/sec/mg tissue)

(B) Diabetes

(C) Vas2870-Inhibitable \( \text{O}_2^\cdot \) (RLU/sec/mg tissue)

(D) p22^{phox}/GAPDH (fold change vs control)

(E) GTP-Rac1/Total Rac1 (fold change vs control)

(F) Membrane Rac1/GAPDH (fold change vs control)

(G) NADPH-Stimulated \( \text{O}_2^\cdot \) (RLU/sec/mg tissue)

(H) Vas2870-Inhibitable \( \text{O}_2^\cdot \) (RLU/sec/mg tissue)

(I) p22^{phox} protein/GAPDH (fold change vs control)
Figure 6

(A) Membrane p47^{phox}/GAPDH

(B) Membrane Rac1/GAPDH

(C) NADPH-Stimulated O$_2^-$ (RLU/sec/mg tissue)

(D) Vas2870-Inhibitable O$_2^-$ (RLU/sec/mg tissue)

(E) Rel. Gene Expr. in IMAs (Adj. to GAPDH)

(F) p-Akt / t-Akt (fold change vs. control)

(G) p-Akt / t-Akt (fold change vs. control)

(H) p-Akt / t-Akt (fold change vs. control)
**Figure 7**

(A) IMA obtained with its PVAT

(B) 4-HNE protein adducts

(C) 4-HNE protein adducts

(D) ADIPOQ relative gene expr in PVAT (fold changes)

(E) PPAR-γ relative gene expr in PVAT (fold changes)

---

**Explanation:**

- **Panel A:** Shows the experimental setup with IMA obtained with its PVAT, indicating the use of NADPH 100μM, +PEG-SOD, and 18 h ex vivo culture.

- **Panel B:** Illustrates 4-HNE protein adducts with a diagram indicating the molecular weight (kD) of proteins and their fold changes.

- **Panel C:** Depicts a similar 4-HNE protein adducts chart with specific markers for IMA1, IMA2, IMA3, and IMA4.

- **Panel D:** Presents a bar chart for ADIPOQ relative gene expression in PVAT with fold changes, showing significant differences between conditions.

- **Panel E:** Displays a bar chart for PPAR-γ relative gene expression in PVAT with fold changes, highlighting the impact of 4HNE and T0070907 under different conditions.

---

**Legend:**

- **IMA +**
- **NADPH -**
- **PEG-SOD -**
- **Low**
- **High**
- **No T2DM**
- **T2DM**

**Significance Levels:**

- **p < 0.01** (***)
- **p < 0.001** (***)
- **p < 0.0001** (***)

---

**Notes:**

- The experimental conditions and results are indicated through various visual and textual elements to convey the data clearly.

---

**Analysis:**

By examining the figures, it is evident that NADPH-stimulated vascular O$_2^·$ activates different pathways in IMAs (IMA1, IMA2, IMA3, IMA4) with subsequent effects on gene expression (ADIPOQ and PPAR-γ) and protein adduct formation (4-HNE) under different conditions (No T2DM vs. T2DM). The use of NADPH 100μM and PEG-SOD influences these outcomes, demonstrating the complexity of metabolic and oxidative stress responses in these contexts.
Online Supplemental Data

**Figure S1.** High molecular weight (HMW) adiponectin released from thoracic adipose tissue (Th-AT) was negatively associated with NADPH-stimulated superoxide (O$_2^-$) generation in the wall of the internal mammary artery (IMA) from the cohort in Study 1. Values are presented as medians [25$^{th}$-75$^{th}$ percentiles].
Figure S2. Internal mammary arteries (IMAs) from patients with type 2 diabetes (T2DM, n=4) exhibited higher levels of malonyldialdehyde (MDA) protein adducts compared to patients without diabetes (No T2DM, n=5), suggesting increased vascular oxidative stress (Panel A). Similarly, total antioxidant capacity in IMAs from patients with T2DM (n=4) was lower compared to IMAs from patients without T2DM (n=4), showing a depletion of endogenous antioxidant systems (Panel B) (n=8). Conversely, incubation of perivascular
adipose tissue (PVAT) with MDA (1 mmol/L) for 16 hours did not significantly alter the
eexpression of adiponectin gene (ADIPQ) (Panel C, n=13). Values are presented as
means±SEM. *P<0.05 versus No T2DM.