Reciprocal Effects of Systemic Inflammation and Brain Natriuretic Peptide on Adiponectin Biosynthesis in Adipose Tissue of Patients With Ischemic Heart Disease


Objective—To explore the role of systemic inflammation in the regulation of adiponectin levels in patients with ischemic heart disease.

Approach and Results—In a cross-sectional study of 575 subjects, serum adiponectin was compared between healthy subjects, patients with coronary artery disease with no/mild/severe heart failure (HF), and patients with nonischemic HF. Adiponectin expression and release from femoral, subcutaneous and thoracic adipose tissue was determined in 258 additional patients with coronary artery bypass grafting. Responsiveness of the various human adipose tissue depots to interleukin-6, tumor necrosis factor-α, and brain natriuretic peptide (BNP) was examined by using ex vivo models of human fat. The effects of inducible low-grade inflammation were tested by using the model of Salmonella typhi vaccine-induced inflammation in healthy individuals. In the cross-sectional study, HF strikingly increased adiponectin levels. Plasma BNP was the strongest predictor of circulating adiponectin and its release from all adipose tissue depots in patients with coronary artery bypass grafting, even in the absence of HF. Femoral AT was the depot with the least macrophages infiltration and the largest adipocyte cell size and the only responsive to systemic and ex vivo proinflammatory stimulation (effect reversible by BNP).

Conclusions—This study demonstrates the regional variability in the responsiveness of human adipose tissue to systemic inflammation and suggests that BNP (not systemic inflammation) is the main driver of circulating adiponectin in patients with advanced atherosclerosis even in the absence of HF. Any interpretation of circulating adiponectin as a biomarker should take into account the underlying disease state, background inflammation, and BNP levels. (Arterioscler Thromb Vasc Biol. 2014;34:2151-2159.)

Key Words: adiponectin • adipose tissue • heart failure • inflammation • natriuretic peptide, brain

Adiponectin is abundantly secreted from adipose tissue (AT), and it plays a role in glucose metabolism and obesity-related insulin resistance.1-3 In human atherosclerosis, adiponectin suppresses vascular oxidative stress and has an antiatherogenic potential.4,5 However, the mechanisms controlling its biosynthesis in the various AT depots and determining its circulating levels are unclear, whereas its clinical value as a biomarker is controversial.

In prospective studies, increased serum adiponectin has been associated with lower risk of developing diabetes mellitus and cardiovascular disease (CVD).6 However, the data about the predictive value of adiponectin in coronary artery disease (CAD) are conflicting6-12; that is, in advanced disease states (eg, in ischemic heart failure [HF]), adiponectin flags poor prognosis independently of the underlying metabolic state.6,12 Therefore, a better understanding of the mechanisms controlling adiponectin levels in different CVD states may allow its use as a clinical biomarker and may identify novel therapeutic targets in patients with advanced CVD.

The regulation of adiponectin biosynthesis is complex; in cultured adipocytes, proinflammatory cytokines down-regulate adiponectin (or ADIPOQ) gene expression,14,15 whereas severe inflammation in experimental animal models down-regulates its expression and reduces circulating adiponectin levels.16-20 In experimental models, the interplay of acute and chronic inflammation with adiponectin expression is complex, and the effect of different agents on adiponectin expression and release is context dependent. In the setting of induced inflammation, BNP has been shown to reduce adiponectin expression and release from AT,21,22 and this effect is reversible by BNP administration.22 In the present study, we found that BNP is a strong predictor of circulating adiponectin and its release from all adipose tissue depots in patients with coronary artery disease with no/mild/severe HF, and patients with nonischemic HF. However, the mechanism by which BNP regulates adiponectin expression and release from AT is yet to be elucidated.

Received on: April 15, 2014; final version accepted on: July 14, 2014.

From the Division of Cardiovascular Medicine, Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom (A.S.A., M.M., P.C., J.D., R.P., N.N., T.D.K., R.L., S.N., K.M.C., C.A.); 1st Cardiology Department, Athens University Medical School, Athens, Greece (C.P., M.D., C.B., D.T.); Department of Cardiac Surgery, John Radcliffe Hospital, Oxford, United Kingdom (R.D.S., M.P., R.S.); and NIHR Oxford Musculoskeletal Biomedical Research Unit & Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, United Kingdom (P.W.).

*These authors contributed equally to this article.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.114.303828/-/DC1.

Correspondence to Charalambos Antoniades, MD, PhD, Division of Cardiovascular Medicine, University of Oxford, West Wing L6 John Radcliffe Hospital, Headley Way, Headington, Oxford OX3 9DU, United Kingdom. E-mail antoniadis@well.ox.ac.uk

© 2014 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.114.303828

2151
levels. In humans, however, atherosclerosis-related low-grade inflammation has been associated with both increased and decreased plasma adiponectin, while there is also evidence that advanced, chronic inflammation may even increase adiponectin levels. These conflicting findings suggest that mechanisms related with the underlying disease state may override the regulatory role of inflammation on adiponectin biosynthesis in advanced CVD states. As such, stimulation of adiponectin synthesis in cultured adipocytes by brain natriuretic peptide (BNP) could explain the increased adiponectin levels in patients with HF despite the significant background inflammation, although this link between BNP and adiponectin biosynthesis has never been demonstrated at the level of human AT.

In this study, we explore the interplay between systemic inflammation and BNP in the regulation of adiponectin expression and release from different human AT depots using ex vivo models of human AT. Using in vivo and ex vivo models of inflammation and a model of inducible low-grade inflammation, we comprehensively characterize the relationship between systemic inflammation and adiponectin levels in humans.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

**Impact of Left Ventricular Function and Circulating BNP on Adiponectin Expression/Release From AT of Patients With Ischemic Heart Disease**

Study design is summarized in Figure 1 and patient demographics in the Table. In study arm 1, we observed that body

---

**Figure 1.** Study design. AT indicates adipose tissue; BNP, brain natriuretic peptide; CABG, coronary artery bypass grafting surgery; CAD, coronary artery disease; Fem-AT, femoral adipose tissue; HF, heart failure; IL-6, interleukin 6; LVEF, left ventricular ejection fraction (by echocardiography); Sc-AT, subcutaneous adipose tissue; Th-AT, thoracic adipose tissue; and TNF-α, tumor necrosis factor-α.

---

**Cross-sectional study of CAD/HF patients (Arm 1)**

- 201 healthy
- 173 CAD with LVEF > 50%
- 135 CAD with LVEF 30-50%
- 33 CAD with LVEF < 30%
- 33 with no CAD and LVEF < 30%

**Patients with ischaemic heart disease: The CABG model (Arm 2)**

- 258 patients undergoing CABG
- Blood samples
- Biopsies of Fem-AT, Th-AT, Sc-AT
- Snap frozen for gene expression studies & immunohistochemistry
- Cultured ex-vivo bioassay

**Studies using an ex vivo model of human AT (Arm 3)**

- 13 patients undergoing CABG
- Biopsies of Fem-AT, Th-AT, Sc-AT
- Ex-vivo incubation for 24h with:
  - TNF-α + IL-6
  - TNF-α + IL-6 + BNP
  - Control
- Gene expression studies & AdN release

**Inducible low-grade inflammation: Model of S. typhi Vaccination (Arm 4)**

- 19 healthy volunteers
- 10 S. typhi Vaccine
- 9 Placebo injection
- Serum and at 0, 8, 12 and 24h
mass index was inversely correlated with circulating adiponectin in healthy adults (Figure 2A) and patients with CAD and preserved left ventricular ejection fraction (Figure 2B), but this association was lost in patients with established left ventricular impairment of ischemic or nonischemic pathogenesis (Figure 2C). Circulating adiponectin levels were not significantly different between patients with CAD with normal or mildly impaired left ventricular ejection fraction and healthy individuals (Figure 2D). However, there was a striking elevation of circulating adiponectin in the presence of ischemic or nonischemic HF as evidenced by impaired left ventricular ejection fraction (Figure 2D). Importantly, plasma levels of high-sensitivity C-reactive protein (hsCRP) were significantly elevated in the patients with CAD compared with healthy controls, and they were further increased in the presence of HF (Table). Interestingly, the significant correlation observed between circulating adiponectin and flow-mediated dilatation, a marker of endothelial function, in the healthy population was weaker in patients with advanced atherosclerosis and preserved left ventricular ejection fraction, and it was lost in patients with ischemic or nonischemic HF (Figure 2E–2H).

To explore the relationship between systemic inflammation and adiponectin levels in atherosclerosis, we focused on a more homogenous group of patients with CAD without evidence of HF undergoing coronary artery bypass grafting (study arm 2). In this cohort, we searched for predictors of circulating adiponectin. In univariate analysis, there was a significant correlation between log(serum adiponectin) and age ($r=0.179$, $P=0.009$), diabetes mellitus (rho=$-0.126$, $P=0.06$), body mass index (BMI) (rho=$-0.126$, $P=0.06$), body mass index (BMI) (rho=$-0.176$, $P=0.015$), female sex (rho=$0.183$, $P=0.007$), and dyslipidemia (rho=$-0.192$, $P=0.005$). Importantly, log(serum adiponectin) was not correlated with either log(serum hsCRP) ($r=-0.125$, $P=0.095$) or log(serum interleukin-6 [IL-6]) ($r=0.089$, $P=0.239$). In a multivariable model where we included all these variables, the independent predictors of log(serum adiponectin) were age ($\beta$ [SE], 0.007 [0.003]; $P=0.006$), female sex ($\beta$ [SE], 0.131 [0.059]; $P=0.028$), body mass index ($\beta$ [SE], $-0.10$ [0.006]; $P=0.084$), diabetes mellitus ($\beta$ [SE], $-0.101$ [0.054]; $P=0.062$), and dyslipidemia ($\beta$ [SE], $-0.128$ [0.048]; $P=0.008$). Given that this model was built only with demographic/anthropometric characteristics of the participants, we then searched for additional biomarkers that could predict circulating adiponectin. We quantified plasma BNP, and we observed a significant association between log(plasma BNP) and log(serum adiponectin) ($r=0.282$, $P<0.0001$). When log(plasma BNP) was included into the multivariable model, it became clear that this was the strongest independent predictor of log(serum adiponectin) with ($\beta$ [SE], 0.153 [0.05]; $P=0.003$), even in the absence of clinical HF. Importantly, circulating BNP was significantly associated with both the expression (Figure 3A–3C) and the release (Figure 3B–3D in the online-only Data Supplement) of adiponectin from all 3 types of AT examined in this population (thoracic adipose tissue [Th-AT], subcutaneous adipose tissue [Sc-AT], and femoral adipose tissue [Fem-AT]), and further supporting the notion that in human atherosclerosis, circulating BNP (rather than systemic inflammation) is the key driver of adiponectin expression in all AT depots and controls its release in the circulation even in the absence of HF syndrome.

### Table. Demographic Characteristics of the Participants

<table>
<thead>
<tr>
<th>Arm 1</th>
<th>Arm 2</th>
<th>Arm 3</th>
<th>Arm 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-Sectional Study of Patients With CAD/HF</td>
<td>Patient With CABG</td>
<td>Ex Vivo Studies</td>
<td>Inducible Low-Grade In Vivo Inflammation</td>
</tr>
<tr>
<td>Healthy Controls</td>
<td>CAD, EF&gt;50%</td>
<td>CAD, EF 30–50%</td>
<td>CAD, EF&lt;30%</td>
</tr>
<tr>
<td>Participants, n (men)</td>
<td>201 (171)</td>
<td>173 (144)</td>
<td>135 (123)</td>
</tr>
<tr>
<td>Age, y</td>
<td>66±0.7</td>
<td>65±0.7</td>
<td>64±0.9</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>47</td>
<td>70</td>
<td>65</td>
</tr>
<tr>
<td>Dyslipidemia, %</td>
<td>24</td>
<td>64</td>
<td>67</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.4±0.3</td>
<td>27.6±0.4</td>
<td>27.7±0.5</td>
</tr>
<tr>
<td>hsCRP, mg/L*</td>
<td>0.9 (0.5–1.8)</td>
<td>1.5 (0.8–3.4)†</td>
<td>1.8 (0.8–4.10)†</td>
</tr>
<tr>
<td>Medication, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEi/ARBs</td>
<td>17/13</td>
<td>53/12</td>
<td>58/15</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>6.5</td>
<td>78</td>
<td>80</td>
</tr>
<tr>
<td>Aspirin</td>
<td>1</td>
<td>80</td>
<td>81</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>0.5</td>
<td>32</td>
<td>43</td>
</tr>
<tr>
<td>Statins</td>
<td>11</td>
<td>84</td>
<td>84</td>
</tr>
<tr>
<td>CCBs</td>
<td>7.5</td>
<td>32</td>
<td>21</td>
</tr>
</tbody>
</table>

Values expressed as mean±SEM. ACEi indicates angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; BMI, body mass index; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CCBs, calcium channel blockers; DM, diabetes mellitus; EF, ejection fraction; HF, heart failure; and hsCRP, high-sensitivity C-reactive protein.

*Values expressed as median (25th–75th percentile).

†$P<0.001$; ‡$P<0.01$ vs healthy controls.
Atherosclerosis-Related Inflammation and Biosynthesis of Adiponectin From AT of Patients With Ischemic Heart Disease

Given that classic inflammatory biomarkers (circulating hsCRP and IL-6) were not significant predictors of circulating adiponectin, we quantified the local expression of ADIPOQ gene in the human AT biopsies where we observed that circulating IL-6 was inversely related with the ADIPOQ gene expression (Figure 3D–3F) and adiponectin protein release (Figure IF–IH in the online-only Data Supplement) only in Fem-AT but not in Th-AT or Sc-AT. Similarly, circulating monocyte chemoattractant protein 1 was inversely related with ADIPOQ gene expression and adiponectin protein release (Figure II in the online-only Data Supplement) only in Fem-AT but not in Th-AT or Sc-AT. To understand the regulatory mechanisms responsible for ADIPOQ gene expression in these AT depots, we quantified the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ, a known upstream regulator of adiponectin in cultured adipocytes), and we observed the same result: high circulating IL-6 was related with low PPAR-γ gene expression only in Fem-AT but not in Th-At or Sc-AT (Figure 3G–3I). Importantly, the correlation between PPAR-γ and ADIPOQ gene expression was significantly stronger in Fem-AT compared with either Sc-AT (z score, −3.846; \(P=0.00012\)) or Th-AT (z score, −0.513; \(P=0.00001\)). To validate this result biologically, we used the expression of CD36 as the positive control, as CD36 expression is known to be regulated predominantly by PPAR-γ, and we demonstrated that the correlation between PPAR-γ and CD36 gene was similar across all AT depot types (Figure 3J–3L).

To explain the regional variability in the responsiveness of human AT to systemic low-grade inflammation, we performed immunohistochemistry of Th-AT, Sc-AT, and Fem-AT sections from 7 consecutive patients, and we observed significantly lower infiltration of Fem-AT by CD68+ cells (macrophages) compared with Sc-AT (intermediate infiltration) and Th-AT (highest infiltration) as demonstrated in Figure 4B and 4J to 4L. This was also confirmed by quantifying CD68 mRNA levels in all three AT depot types in the population included in study arm 2, where there was a 3-fold difference in CD68 gene expression in Th-AT compared with Fem-AT and a 2-fold difference between Sc-AT and Fem-AT (Figure 4A). Interestingly, the
average adipocyte cell size was significantly lower in Th-AT compared with Fem-AT, with Sc-AT having intermediate-size adipocytes (Figure 4C and 4G–4I). We then assessed macrophages’ M1/M2 polarization in the various AT depots. In the population of study arm 2, we quantified gene expression of CCR7 (C-C chemokine receptor type 7 or CD107, marker of M1 macrophages) and CD206 (mannose receptor C type 1 or CD206, marker of M2 macrophages). The CCR7/CD206 gene expression ratio in Th-AT was ≈3-fold higher than Sc-AT and ≈5-fold higher than Fem-AT (Figure 4D). By
using immunohistochemistry, we then stained AT sections for CD40+ (surface marker of M1 macrophages) and CD206+ (surface marker of M2 macrophages), and we observed that the ratio of CD40+/CD206+ cells in Th-AT was ≈2-fold higher than Sc-AT and ≈5-fold higher than Fem-AT (Figure 4E and 4M–4R). Interestingly, IL-6-receptor gene expression was significantly lower in Th-AT or Sc-AT compared with Fem-AT (Figure 4F).

These morphological differences could explain the different responsiveness of Fem-AT depot to exogenous (circulating) inflammatory stimulation in vivo. To further investigate the role of inflammation as a regulator of adiponectin release from human AT in the presence or absence of BNP, we performed an ex vivo study where AT from all three depots (Sc-AT, Th-AT, and Fem-AT) was exposed to IL-6 (25 ng/mL) and tumor necrosis factor-α (4 ng/mL)±BNP (1 nmol/L) for 24 hours. Inflammatory stimulation suppressed the expression of PPAR-γ, CD36, and ADIPOQ gene, resulting into a decrease of adiponectin release in AT culture supernatants only in Fem-AT, an effect that was reversed by BNP (Figure 5A–5D). Importantly, none of the other AT depots (Sc-AT or Th-AT) responded to exogenous inflammatory stimulation (Figure 5A–5D).

Figure 4. Study arm 2: differences in the inflammatory profile of different adipose tissue depots. A, There was significantly higher CD68 gene expression (expressed predominantly by infiltrating macrophages) in thoracic adipose tissue (Th-AT) compared with either subcutaneous adipose tissue (Sc-AT) or femoral adipose tissue (Fem-AT). In immunohistochemistry with anti-CD68, Th-AT had significantly lower adipocyte size (C, G–I) and higher infiltration by CD68+ macrophages (B, J–L) compared with Sc-AT or Fem-AT. Moreover, Th-AT had significantly higher ratio of CCR7 (marker of M1 macrophages)/CD206 (marker of M2 macrophages) gene expression compared with Sc-AT and Fem-AT (D). Similarly, after staining for CD40+ (surface marker of M1 macrophages, M–O) and CD206+ (surface marker of M2 macrophages, P–R), the CD40+/CD206+ ratio was higher in Th-AT compared with Sc-AT and Fem-AT (E). F, Th-AT had also significantly lower expression of interleukin-6 receptor compared with the other two depots. Gene expression studies were performed in the entire population of study arm 2 and immunohistochemistry in sets of tissue obtained from 7 patients. Values are expressed as median (25th–75th percentile) for A–C and F or mean±SEM for panels D and F. P values were calculated by 1-way repeated-measures ANOVA followed by paired-t test vs Th-AT on the log-transformed values followed by Bonferroni correction. *P<0.05; **P<0.01 vs Th-AT.

Effects of Systemic Inflammation on Adiponectin Levels: Insights From a Clinical Model of Inducible Low-Grade Inflammation
To further investigate the effects of systemic low-grade inflammation on adiponectin levels, we used a clinical model
of inducible in vivo inflammation: we randomized 19 healthy young individuals to receive either Salmonella typhi (S. typhi) vaccine (as a means to induce systemic low-grade inflammation) or placebo (normal saline injection) and quantified the changes of circulating adiponectin in parallel to the respective changes of circulating inflammatory markers (IL-6 and hsCRP), at 0, 8, 12, and 24 hours (study arm 4). As we expected from our previously published work on this model,23 S. typhi vaccination induced a low-grade inflammatory response (Figure 5E–5F) and significantly reduced serum adiponectin when compared against placebo (Figure 5G) independently of any changes in BNP (Figure 5H).

These findings suggest that systemic low-grade inflammation reduces circulating adiponectin levels in healthy individuals where BNP levels are low, hence biologically nonrelevant.

**Discussion**

Using a range of ex vivo and in vivo approaches, we explored the mechanisms regulating adiponectin biosynthesis in patients with ischemic heart disease. We demonstrate that plasma BNP is the key determinant of circulating adiponectin and a major driver of adiponectin release from all AT depots of patients with ischemic heart disease. On the contrary, low-grade inflammation observed in CAD is not a significant regulator of circulating adiponectin in this population, as circulating inflammatory biomarkers predict ADIPOQ gene expression and release only in selected AT depots with low infiltration with macrophages (such as femoral AT) but not in highly inflamed depots (ie, thoracic AT). This could also be explained by the stronger effect of PPAR-γ signaling on the regulation of ADIPOQ gene expression in the femoral compared with other fat depots. We then used ex vivo models of human AT and demonstrated that only femoral (but not thoracic or subcutaneous) AT responds to exogenous inflammatory stimulation by suppressing PPAR-γ signaling and adiponectin expression/release, effects reversed by BNP. Induction of systemic low-grade inflammation in healthy individuals by using the model of S. typhi vaccination reduced circulating adiponectin levels when compared with placebo. These findings suggest that circulating adiponectin levels are regulated by complex interactions between cytokines and BNP, and the net effect is determined by the background disease state and the nature of the underlying inflammatory stimulus.

Adiponectin is an adipokine with well-established insulin-sensitizing effects, and it is protective against the development of diabetes mellitus.24 Despite its potential antiatherogenic effects, circulating adiponectin has not been established as a biomarker in CVD because of the conflicting data on its clinical predictive value; although the first evidence supported that higher adiponectin predicts low CVD risk in healthy individuals, elevated adiponectin levels have been associated with increased mortality in patients with ischemic heart disease.28 Moreover, in recent meta-analyses, circulating adiponectin has been associated with increased risk for stroke and higher risk for CVD and total mortality in secondary prevention.28 Importantly, in serial studies by Koh et al, it was clearly demonstrated that conventional treatments (eg, statins or angiotensin-converting enzyme inhibitors and others) routinely used in patients with advanced atherosclerosis may also have a significant impact on circulating adiponectin levels. Therefore, despite the beneficial metabolic effects of adiponectin, its value as a biomarker is poorly understood and the mechanisms regulating its circulating levels are unclear. It is possible that low circulating adiponectin may be detrimental for cardiovascular system in obesity, but in advanced CVD states, its levels are increased as a defense mechanism to protect the cardiovascular system, so in these conditions, it may actually flag the underlying advanced CVD.

Studies with cultured 3T3-L1 cells and human primary adipocytes have consistently shown that proinflammatory...
cytokines downregulate adiponectin expression and release, whereas in other studies, natriuretic peptides (which are elevated in HF) have the opposite effect. Despite these experimental findings, the net effect of systemic inflammation and BNP on adiponectin biosynthesis in AT of patients with ischemic heart disease is unclear.

In this study, we demonstrate that BNP is a strong stimulus for adiponectin release from all AT depots (both in vivo and ex vivo), controlling circulating adiponectin in patients with coronary atherosclerosis even in the absence of HF. On the contrary, atherosclerosis-associated low-grade inflammation is not related with changes in circulating adiponectin levels in patients with CAD. Circulating IL-6 and hsCRP are inversely related with ADIPOQ gene expression (possibly via PPAR-γ signaling) only in Fem-AT that highly expresses IL-6 receptor but not in more inflamed AT depots, such as Th-AT or Sc-AT, which are highly infiltrated with macrophages shifted toward the M1 (proinflammatory) rather than M2 (resident) phenotype. Our data suggest that endogenous inflammation of Th-AT and Sc-AT may lead to downregulation of IL-6R in these AT depots, reducing their responsiveness to exogenous IL-6 coming from the circulating pool. Importantly, the correlation between circulating adiponectin and endothelial function observed in healthy individuals was weaker in patients with coronary atherosclerosis and was absent in the presence of HF, suggesting that the value of circulating adiponectin as a biomarker in ischemic heart disease is limited by the confounding effect of circulating BNP and the degree of endogenous inflammation within each AT depot, which limits their ability to respond to systemic inflammation by suppressing adiponectin release.

Although acute inflammation in vitro has well-described negative effects on adiponectin biosynthesis in cultured adipocytes, the effects of acute inflammation in vivo are unclear; in mice, lipopolysaccharide injection lowers ADIPOQ gene expression from white AT, but in patients with diabetes mellitus, short-term infusion of IL-6 increases ADIPOQ gene expression in subcutaneous AT. We induced systemic low-grade inflammation in healthy individuals by using S. typhi vaccine, and we observed that this type of inducible inflammation decreased circulating adiponectin levels within 24 hours compared with placebo, having no effects on circulating BNP. This is in agreement with our observation that systemic inflammation suppresses adiponectin biosynthesis in the noninflamed human Fem-AT. This study provides comprehensive evidence that in humans systemic low-grade inflammation suppresses adiponectin levels and identifies its role as potential link between systemic inflammation and the early stages of the development of CVD.

In conclusion, we demonstrate that low-grade inflammation reduces adiponectin levels in populations without significant CVD and low plasma BNP, explaining why its low levels predict the onset of CVD. However, after the development of advanced CVD, adiponectin levels are no longer negatively controlled by low-grade inflammation, but they are driven upward by circulating BNP levels. Therefore, high adiponectin levels in these populations flag the high levels of circulating BNP, explaining why high circulating adiponectin predicts (indirectly) worse clinical outcome in these populations. Therefore, the interpretation of adiponectin as a biomarker should always take into account the underlying CVD state.

Acknowledgment

We thank Dr. Joshua Chait for technical assistance with the immunohistochemistry protocol and the cell size measuring method.

Sources of Funding

The study was supported by a Marie Curie Intra-European Fellowship from the European Commission, 7th Framework Programme 2007 to 2013 (grant agreement number: 300289, project acronym: HRS-EAT), the British Heart Foundation (FS/11/66/28855 and Oxford CRE: RE/08/004), and the Greek National Strategic Reference Framework through research funding program Heracleitus II.

Disclosures

None.

References

always take into account the underlying CVD state and the levels of brain natriuretic peptide. Adiponectin biosynthesis driving its levels in patients with advanced CVD. Therefore, any interpretation of adiponectin as a biomarker should take into account its responsiveness to inflammatory stimulation. On the contrary, the elevated circulating brain natriuretic peptide leads to a global increase of inflammation, and vaspin levels in rheumatoid arthritis.


Significance

Obesity is a cardiovascular risk factor, and adipose tissue affects both cardiac and vascular physiology. Circulating adiponectin, one of the main antiatherogenic adipokines secreted by human adipose tissue, has been paradoxically associated with adverse clinical outcome in patients with established cardiovascular disease (CVD). We now provide the first thorough investigation of the mechanisms regulating adiponectin biosynthesis directly in the human adipocyte tissue in different CVD states. We first demonstrate that circulating adiponectin in healthy individuals is suppressed by systemic inflammation, but in advanced CVD states, adipose tissue infiltration by inflammatory cells eliminates its responsiveness to inflammatory stimulation. On the contrary, the elevated circulating brain natriuretic peptide leads to a global increase of adiponectin biosynthesis driving its levels in patients with advanced CVD. Therefore, any interpretation of adiponectin as a biomarker should always take into account the underlying CVD state and the levels of brain natriuretic peptide.
Reciprocal Effects of Systemic Inflammation and Brain Natriuretic Peptide on Adiponectin Biosynthesis in Adipose Tissue of Patients With Ischemic Heart Disease

Arterioscler Thromb Vasc Biol. 2014;34:2151-2159; originally published online July 24, 2014; doi: 10.1161/ATVBAHA.114.303828

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/9/2151

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Detailed Methods

Population and Protocol of Cross-Sectional Study With CAD/HF Patients (Arm 1)

To examine the role of systemic inflammation as a regulator of AdN levels in advanced atherosclerosis with/without HF, we designed a cross-sectional study which included 575 subjects: 341 patients with angiographically documented CAD, 201 healthy individuals without any history or clinical signs of CAD and 33 patients with clinical syndrome of HF but without CAD (documented by coronary angiography) (Figure 1 and Table). Exclusion criteria were any inflammatory, infectious, liver / renal disease or malignancy. Patients receiving non-steroidal anti-inflammatory drugs as well as patients with unstable angina event(s) during the last 6 weeks were also excluded. Blood samples were obtained as part of the study and left ventricular ejection fraction (LVEF) was estimated by echocardiography. Endothelial function was estimated by measuring Flow Mediated Dilatation (FMD) of the brachial artery in subgroups of this population as stated in the legend to supplementary Figure 1.

Population and Protocol of Study with CABG Patients (Arm 2)

The population of Study arm 2 consisted of 258 patients (Figure 1 and Table) undergoing elective coronary artery bypass grafting surgery (CABG). Blood samples were obtained and FMD was evaluated preoperatively.\textsuperscript{1,2} Exclusion criteria were any inflammatory, infectious, liver / renal disease or malignancy. Patients receiving non-steroidal anti-inflammatory drugs were also excluded. During surgery, samples of AT were harvested, i.e. subcutaneous (Sc-AT, from the site of the chest incision), thoracic (Th-AT, from the central thoracic area, attached to the pericardium) and femoral AT (Fem-AT, from the site of vein harvesting in the thigh). AT samples were
snap-frozen for gene expression studies and immunohistochemistry studies, and also used for additional AT culture experiments to determine the biosynthetic rate of AdN, as described below.

**Population and Protocol of Ex-Vivo Study with Human AT (Arm 3)**

To explore the direct impact of inflammatory stimulation in the presence/absence of BNP on the various AT depots in patients with atherosclerosis, we recruited 13 patients undergoing elective CABG, under the same exclusion criteria applied for Study arm 2 (Figure 1 and Table). During surgery, AT samples were harvested (Sc-AT, Th-AT and Fem-AT as described in Study arm 2). These samples were used for *ex-vivo* AT culture experiments, as described below.

**Population and Protocol of the Study with Inducible Low-Grade Inflammation In-Vivo (Arm 4)**

To further explore the causal association between systemic inflammation and circulating AdN, we randomised 19 healthy young individuals to receive either *S. typhi* vaccine (as a model of inducible systemic low-grade inflammation, n=10) or placebo (normal saline injection as placebo, n=9). Blood samples were obtained at baseline, 8, 12 and 24 hours post-intervention as shown in Figure 1. All studies had been approved by the Institutional Ethics Review Committee and the subjects gave written informed consent.

**Blood Sampling and Measurements of Circulating Biomarkers**

Serum AdN and IL-6 were measured by enzyme linked immunosorbent assay (ELISA) kits (from BioVendor and R&D Systems respectively) in fasting venous blood samples. Plasma monocyte chemoattractant protein 1 (MCP-1) was also measured by ELISA (from R&D Systems, U.S.A
respectively). hsCRP was measured by the high-sensitivity latex enhanced immunoturbidimetric assay (ADVIA, Bayer HealthCare LLC). Plasma BNP was quantified by chemiluminescent-microparticle immunoassay (Architect BNP, Abbott, Germany).

**Adipose Tissue Culture (Arm 2)**

Samples of Sc-AT, Th-AT and Fem-AT obtained from CABG patients in study arm 2, were used to estimate the biosynthetic rate of AdN, in an *ex vivo* bioassay as we have previously described. After culturing the tissue for 4h, culture supernatants were collected, filtered and stored at -80°C until analysis. This allowed the generation of a unique bio-resource of AT culture supernatants from patients undergoing CABG (as part of the Oxford CABG Bio-Resource). AdN levels were then quantified in AT culture supernatants by using a high sensitivity ELISA kit (BioVendor, Czech Republic).

**Ex-Vivo Experiments with Adipose Tissue (Arm 3)**

In the individual experiments for study arm 3, AT was collected and processed as described above. After the equilibration period AT samples were incubated in the presence or absence of IL-6 (25ng/mL) and TNF-α (4ng/mL) ± BNP (1nM) under the same conditions for 24h. The concentration of BNP was chosen as to be comparable to the maximum circulating BNP levels in severe congestive heart failure. The concentrations of IL-6 and TNF-α were chosen to be 10 fold higher than their estimated intra-tissue concentrations *ex vivo*, and were comparable to the levels we used in our previous *ex vivo* experiments with human tissue in the past. AT culture supernatants were collected, filtered and stored at -80°C until analysis for AdN levels. Incubated AT samples were also used to determine *ADIPOQ* gene expression.
RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Samples of Sc-AT, Th-AT, and Fem-AT were snap frozen in QIAzol (Qiagen, Stanford, CA) and stored at -80°C until processed. RNA was extracted by using the RNeasy Micro or Mini kit (Qiagen). Ribonucleic acid was converted into complementary DNA (Quantitect Rev. Transcription kit - Qiagen), then subjected to qPCR using TaqMan probes for ADIPOQ gene (Life Technologies, Foster City, CA; Assay ID ADIPOQ: Hs00605917_m1), CD68 (Life Technologies; Assay ID CD68: Hs02836816_g1), CD206 (MRC1) (Life Technologies, Assay ID MRC1: Hs00267207_m1), CCR7 (Life Technologies, Assay ID CCR7: Hs01013469_m1), IL6R (Life Technologies, Assay ID IL6R: Hs01075666_m1) and cyclophilin as house-keeping gene (Life Technologies; Assay ID PPIA: Hs04194521_s1). The reactions were performed in triplicate in 384-well plates, using 5ng of cDNA per reaction, on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The efficiency of the reaction in each plate was determined based on the slope of the standard curve; expression of AdN relative to cyclophilin was calculated using the Pfaffl method.4

Immunohistochemistry

AT samples for immunohistochemistry were stored at -80°C in optimal cutting temperature (OCT) media. Briefly, 15 micron sections were stained for CD68/CD40/CD206 expression with Monoclonal Mouse Anti-Human CD68, Monoclonal Mouse Anti-Human CD40 and Monoclonal Anti-Human CD206 antibodies respectively according to the manufacturer’s instructions (Dako CytoMation, Carpinteria, California, USA; AbD Serotec, Kidlington, UK). Non-specific antigen
binding was blocked using serum-free protein block for 1-2 h (#X0909, Dako CytoMation, Carpinteria, California, USA). Sections were incubated with primary antibody (1:50) overnight at 4°C, washed with PBS-T and incubated with secondary antibody (Vectstain ABC-AP Kit (mouse IgG): Vector Laboratories, #PK-6200) for 1 hour at room temperature. The staining was developed using the DAB Substrate kit for Peroxidase (#SK-4100, Vector Laboratories, Burlingame, California, USA) according to the manufacturer’s protocol. After counter-staining with haematoxylin the slides where mounted with Neo-Mount (#109016, Merck KGaA, Darmstadt, Germany) and examined under the microscope. Macrophage infiltration and cell size were quantified by an unbiased observer. Cell size was measured using the software Image J. For each patient 3 different fields were quantified per fat depot at 100x magnification for macrophage infiltration and 200x for cell size.

Assessment of Endothelial Function

Vascular endothelial function was evaluated non-invasively by measuring the flow mediated dilatation (FMD) and endothelium-independent vasodilatation of the brachial artery by ultrasound. This was achieved by using a linear array transducer and automated off-line analysis (Vascular Analyser, Medical Imaging Applications LLC), as previously described.\textsuperscript{1,2} Briefly, brachial artery diameter was recorded before, and sixty seconds after a five minutes forearm blood flow occlusion. A further measurement was made three minutes after administration of a sublingual spray of glyceryl trinitrate 400 µg. FMD and endothelium-independent dilatation (EID) of the brachial artery were defined as the %change in vessel diameter after forearm ischaemia or sublingual glyceryl trinitrate administration, respectively.
**Statistical Analysis**

Continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test. Non-normally distributed variables were log-transformed for analysis and are presented as median [25\textsuperscript{th}-75\textsuperscript{th} percentile] or on log-transformed scale. Normally distributed variables are presented as mean±SEM.

Comparisons between groups in study arm 1 were performed by using one-way ANOVA followed by Bonferroni post-hoc correction for individual comparisons vs healthy control group.

Continuous variables were compared between 3 groups in Study arm 2 using one-way ANOVA. For comparisons of variables between 3 different AT depots from the same patient, repeated measures ANOVA was used followed by paired-t test and Bonferroni correction as described in the respective Figure legends. In Study arm 3 (ex vivo studies), changes of AdN release in culture supernatants or AdN/PPAR-γ/CD36 gene expression in the tissue were evaluated by using two-way ANOVA for repeated measures with [AT type] x [treatment group] interaction, followed by paired samples t-test for individual paired comparisons of each intervention vs control. In Study arm 4, the changes in the reported circulating biomarkers were assessed by two-way ANOVA for repeated measures with [time] x [group] interaction.

Categorical variables were compared by using chi-square test, as appropriate. Correlations between continuous variables were assessed by using bivariate analysis, and Pearson’s coefficient was estimated. When log-transformation of a non-normally distributed variable was not possible (i.e. FMD- see Supplementary Figure 1), the Spearman rho correlation coefficient was calculated. Comparisons of the correlation coefficients between groups were performed by calculating z-score.
In study arm 2, linear regression was performed by using log(serum AdN) as dependent variable, and as independent variables those of the clinical demographic characteristics (age, gender, diabetes, smoking, dyslipidemia, hypertension, body mass index (BMI)) or circulating biomarkers [log(serum IL-6), log(plasma hsCRP) and log(plasma BNP)] that showed an association with the dependent variable at the level of 15%. All statistical tests were performed using SPSS v20.0 and P<0.05 was considered statistically significant.
Supplemental data

Effects of medication on circulating AdN

Circulating AdN was related with the use of ACEi (rho=0.203, P=0.03 in healthy individuals in arm 1 and rho=0.150, P=0.037 in the patients with advanced CABG undergoing CABG in arm 2), the use of beta blockers (rho=-0.133, P=0.03 in CAD with no heart failure arm 1 and rho=-0.272, P=0.0001 in CAD with no heart failure undergoing CABG arm 2), and the use of statins (rho=-0.170, P=0.004 in CAD with no heart failure arm 1, rho=-0.162, P=0.05 in CAD with heart failure in arm 1, rho=-0.381, P=0.024 in patients without CAD but with heart failure in arm 1 and rho=-0.280, P=0.0001 in CAD with no heart failure undergoing CABG in study arm 2).
Supplemental Figure I: Relationships between circulating levels/tissue release of adiponectin and Brain natriuretic peptide or systemic inflammation in Study arm 2. Increased circulating Brain natriuretic peptide (BNP) levels were related with higher circulating adiponectin (AdN, A) as well as with higher release of AdN in the tissue culture supernatants after 4 hours of ex vivo incubation of thoracic (Th-AT, B), subcutaneous (Sc-AT, C) or femoral (Fem-AT, D) adipose tissue. On the contrary, circulating interleukin 6 (IL-6) was not related to circulating AdN (E) or AdN released from Th-AT (F) or Sc-AT (G) after 4 hours of ex vivo incubation. Circulating IL-6 was inversely related with AdN ex-vivo release from the Fem-AT. Values expressed as median [25th-75th percentile]; P-values calculated by one-way ANOVA for multiple comparisons on the log-transformed values across the three tertiles.
Supplemental Figure II. There was no significant association between circulating MCP-1 and either the expression or ADIPOQ gene or the release of adiponectin (AdN) from thoracic (Th-AT, A, D) or subcutaneous (Sc-AT, B, E) adipose tissue in the population of study 2. However, circulating MCP-1 was inversely associated both with ADIPOQ expression and AdN release from femoral (Fem-AT, C, F) adipose tissue. Moreover, there was no significant association between circulating levels of MCP-1 and circulating AdN. Values expressed as median [25th–75th percentile]; P-values calculated by one-way ANOVA for multiple comparisons on the log-transformed values across the three tertiles.
Supplemental References


