Epicardial Adipokines in Obesity and Coronary Artery Disease Induce Atherogenic Changes in Monocytes and Endothelial Cells

Kalypso Karastergiou, Ian Evans, Nicola Ogston, Nazar Miheisi, Devaki Nair, Juan-Carlos Kaski, Marjan Jahangiri, Vidya Mohamed-Ali

Objective—To investigate the hypothesis that release of adipokines by epicardial adipose tissue (EAT) is dysregulated in obesity and/or coronary artery disease (CAD), along with the previously documented expansion of the tissue, and that these molecules induce pathophysiologic changes in human monocytes and coronary artery endothelial cells.

Methods and Results—Adipokines may mediate the effects of adipose tissue on the vasculature. In white nondiabetic patients with CAD (n=62) or without CAD (control group) (n=32), subdivided by a body mass index (calculated as weight in kilograms divided by height in meters squared) of 27 or less and greater than 27, 13 cytokines were identified by protein array analysis as EAT products. Interleukin 6, interleukin 8, monocyte chemoattractant protein 1, plasminogen activator inhibitor 1, growth-related oncogene-α, and macrophage migration inhibitory factor were the most abundant. Adiponectin release was suppressed in patients with obesity and CAD, and regulated on activation T-cell and secreted was induced in patients with CAD. EAT-conditioned media induced migration of monocytes tryptophan hydroxylase 1 cells, an effect exacerbated in those with CAD. Moreover, conditioned media from patients with CAD and a body mass index of greater than 27 increased the adhesion of tryptophan hydroxylase 1 cells to human monocytes and coronary artery endothelial cells by 15.1% (P=0.002) and expression of intercellular adhesion molecule 1 by 2.8-fold (P=0.002). This effect was reversed by recombinant adiponectin.

Conclusion—EAT products are altered in both obesity and CAD and induce atherogenic changes in relevant target cells. (Arterioscler Thromb Vase Biol. 2010;30:00-00.)

Key Words: obesity ■ adipokines ■ adhesion molecules ■ endothelial cells ■ monocytes

Epidemiological data have established a close association between obesity and increased cardiovascular morbidity and mortality.1 Adipokines, the secretory products of adipose tissue, represent a novel and causative link between obesity and atherosclerosis. Adiponectin, leptin, interleukin (IL) 6, tumor necrosis factor α, and plasminogen activator inhibitor (PAI) 1 all affect vessel wall homeostasis.2

The adipose organ is dispersed, and its effects on the vasculature depend on its abundance and location. Thus, although the large subcutaneous adipose tissue (SAT) depots mostly determine circulating levels of adipokines, perivascular depots, such as the epicardial adipose tissue (EAT) around the coronary arteries, are likely to have a direct impact on atherosclerosis as the result of anatomical proximity. Indeed, periadventitial adiponectin or proinflammatory cytokines can affect the atherosclerotic process in vivo.3,4 After reaching the intima either by diffusion across the vessel wall or via the vasa vasorum,5,6 EAT has recently been investigated,7 and imaging studies8,9 have described its size and distribution in large populations. Its metabolic and secretory functions are thought to be similar to visceral adipose tissue, with which it shares common embryonic origin.10 Both proinflammatory/proatherogenic and anti-inflammatory/atheroprotective molecules are expressed in EAT at levels that usually differ from the subcutaneous depot.7,11–13 They are associated with the presence and extent of coronary artery disease (CAD),13–15 whereas the significance of obesity remains unclear. However, data on the expression of these molecules, mostly at the RNA level and less at the protein level, do not prove causality. For EAT to be significant, it has to actively release molecules at levels that can induce relevant biological effects.

Therefore, the aims of this study were as follows: (1) to characterize adipokine release by EAT from white nondiabetic patients and (2) to investigate whether these molecules induce pathophysiological changes in human monocytes and/or coronary artery endothelial cells (HCAECs), dependent on obesity and CAD.
Table 1. Patient Characteristics*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control Group</th>
<th>CAD Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMI ≤27 (n=20)</td>
<td>BMI &gt;27 (n=12)</td>
</tr>
<tr>
<td>Age, y‡</td>
<td>69.5 (60.5–75.0)</td>
<td>64.5 (62.0–73.5)</td>
</tr>
<tr>
<td>Male sex</td>
<td>45.0</td>
<td>58.3</td>
</tr>
<tr>
<td>BMI‡</td>
<td>24.5 (22.0–25.4)</td>
<td>31.8 (29.0–37.5)</td>
</tr>
<tr>
<td>Waist circumference, cm‡</td>
<td>90.2±12.2</td>
<td>113.0±13.0</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic§</td>
<td>139±28</td>
<td>129±30</td>
</tr>
<tr>
<td>Diastolic‡</td>
<td>75 (65–80)</td>
<td>75 (70–90)</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>70.0</td>
<td>75.0</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>55.0</td>
<td>67.0</td>
</tr>
<tr>
<td>Smoking status, ex/current</td>
<td>60.0/10.0</td>
<td>50.0/0.0</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>33.0</td>
<td>36.0</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>25.0</td>
<td>33.0</td>
</tr>
<tr>
<td>Statin</td>
<td>55.0</td>
<td>33.0</td>
</tr>
<tr>
<td>ACEI/ARB</td>
<td>20.0</td>
<td>58.0</td>
</tr>
<tr>
<td>CCB</td>
<td>25.0</td>
<td>33.0</td>
</tr>
<tr>
<td>β-blocker</td>
<td>30.0</td>
<td>33.0</td>
</tr>
<tr>
<td>Extent of CAD vessel disease, 1/2/3¶</td>
<td>0/0/0</td>
<td>0/0/0</td>
</tr>
</tbody>
</table>

ACEI indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); CAD, coronary artery disease; CCB, calcium channel blocker.

*Data are given as percentage of patients unless otherwise indicated.
†P values represent comparisons using a 1-way ANOVA or the Kruskal-Wallis, χ², or Fisher exact test as appropriate.
‡Data are given as median (IQR).
§Data are given as mean±SD.
¶Greater than 75% indicates luminal stenosis; and greater than 50%, left main stem. Left main stem disease was scored as 3-vessel disease.

Methods

Study Population

Patients undergoing elective cardiac surgery (n=94) and those diagnosed as having CAD (n=62) or as not having CAD (control group) (n=32), who were undergoing valve replacement, were recruited and divided into those with a body mass index (BMI: calculated as weight in kilograms divided by height in meters squared) of 27 or less or a BMI of greater than 27. This cutoff was chosen because it corresponds to a threshold for abnormal glucose metabolism and increased mortality.16,17 CAD was defined angiographically as the presence of 1 or more stenoses of greater than 50% of the luminal diameter for the left main stem artery and greater than 75% for the rest of the coronary arteries. Patients of nonwhite origin, diagnosed as having diabetes mellitus, systemic inflammatory/terminal illnesses, and HIV infection or lipodystrophy; or those who underwent recent surgery or were treated with corticosteroids or nonsteroidal anti-inflammatory drugs, other than low-dose aspirin, were excluded. All patients gave informed written consent for the study, which was approved by the local ethics committee.

Body weight, height, and waist circumference at the midpoint between the lowest rib and the upper iliac crest were measured (to the nearest 0.1 kg/0.1 cm) in light clothing without shoes. Blood pressure was measured with a random 0 sphygmomanometer. Demographic data, medical history, family history of CAD, current medications, and smoking habits were recorded.

Blood and Adipose Tissue Sampling

Fasting venous blood samples (20 mL) were collected preoperatively, and serum/plasma was stored at −80°C. Fasting glucose, lipid, hs-C-reactive protein, and insulin levels were assessed; and the Homeostasis Model Assessment Index–IR was calculated as described in the supplemental Material (available online at http://atvb.ahajournals.org).

SAT, 0.5 to 2.0 g, was excised from the site of sternotomy incision; and EAT, 0.4 to 1.0 g, was excised close to the course of the right coronary artery, approximately 45 minutes after the anesthetic and before the initiation of cardiopulmonary bypass.

Adipose Tissue Organ Culture

After removal of visible vessels and fibrous tissue, SAT/EAT samples were finely minced and 2×50 mg were incubated in serum-free medium, 0.5 mL, enriched with BSA, 1 g/L (Cellgro Complete) for 24 hours at 37°C and 5% CO₂. At the end of the incubation, tissue was snap frozen in liquid nitrogen and stored at −80°C along with the culture supernatant. In a subset of patients (n=19), as previously described, the tissue was used for DNA extraction (as a marker of cell numbers) (DNeasy Blood & Tissue kit; Qiagen). Tissue responsiveness to hormones was examined with isoprenaline and comparable numbers (DNeasy Blood & Tissue kit; Qiagen).

Adipokine Assays

A cytokine protein array (Human Cytokine Array Panel A; R&D Systems) was used to analyze EAT-conditioned media from representative patients (n=19), as previously described.2 Total levels of adiponectin, leptin, monocyte chemoattractant protein (MCP) 1, PAI-1, regulated on activation T-cell and secreted (RANTES), soluble IL-6 receptor (sIL-6R), growth-related oncogene α (GROα), and soluble intercellular adhesion molecule (sICAM) 1 were determined in serum and EAT-conditioned media by commercial ELISAs. IL-6 in conditioned media was analyzed by normal-sensitivity ELISA (range, 3 to 500 pg/mL) and in serum
by high-sensitivity ELISA (range, 0.16 to 10.00 pg/mL) (all available from R&D Systems). Adiponectin isoforms were determined in serum as previously described, with small modifications. All interassay and intra-assay coefficients of variation were less than 10%.

**HCAEC and Monocyte Experiments**

HCAECs (TCS Cellworks; Park Leys) and tryptophan hydroxylase (THP) 1 cells, a human monocytic cell line (ATCC), were cultured as described in the supplemental Material. HCAECs were treated with EAT-conditioned or serum-free media for 12 hours; and mRNA expression of vascular cell adhesion molecule (VCAM) 1, ICAM-1, and E-selectin was assessed by quantitative RT-PCR. Unstimulated HCAECs were used to assess adipoR1/2 expression.

HCAECs were treated for 6 hours with EAT-conditioned medium from the 4 groups, and the adhesion assay was performed as previously described, with modifications. The migration of THP-1 cells toward EAT-conditioned media; recombinant MCP-1, 20 ng/mL (positive control); or serum-free media was assessed in a 48-well Boyden chamber, as previously described, after 1 hour of incubation. Details are included in the supplemental Material.

**Gene Expression**

Total RNA was extracted from paired EAT/SAT samples, 100 mg, and HCAECs (RNeasy Lipid Tissue Mini Kit; Qiagen) and 0.5 μg of reverse-transcribed reagents (Taquin Reverse Transcription Reagents; Roche). Transcripts encoding various genes were measured by quantitative RT-PCR (ABI Prism 7900HT Sequence Detection System).
Circulating adipokine levels are shown in supplemental Table I. Circulating metabolic and inflammatory markers are included in the supplemental Table.

Table 2. Predictors of Epicardial Adipokines in Multiple Linear Regression Analysis*

<table>
<thead>
<tr>
<th>Adipokine</th>
<th>Variables Standardized β</th>
<th>P Value</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin Non-CAD</td>
<td>0.218</td>
<td>0.03</td>
<td>1.7 to 33.8</td>
</tr>
<tr>
<td>BMI</td>
<td>−0.333</td>
<td>0.001</td>
<td>−4.2 to −1.1</td>
</tr>
<tr>
<td>Female sex</td>
<td>0.318</td>
<td>0.002</td>
<td>9.8 to 42.3</td>
</tr>
<tr>
<td>RANTES Non-CAD</td>
<td>−0.576</td>
<td>0.008</td>
<td>−163 to −26</td>
</tr>
<tr>
<td>Statin</td>
<td>−0.367</td>
<td>0.08</td>
<td>−132 to 8.7</td>
</tr>
<tr>
<td>IL-6 Non-CAD</td>
<td>−0.323</td>
<td>0.03</td>
<td>−9105 to −367</td>
</tr>
<tr>
<td>MCP-1 Age</td>
<td>0.232</td>
<td>0.09</td>
<td>−0.02 to 0.27</td>
</tr>
<tr>
<td>Statin</td>
<td>−0.409</td>
<td>0.004</td>
<td>−7.3 to −1.5</td>
</tr>
</tbody>
</table>

BM indicates body mass index (calculated as weight in kilograms divided by height in meters squared); CAD, coronary artery disease; IL, interleukin; MCP, monocyte chemotactic protein; RANTES, regulated upon activation T-cell chemokine.

*Variables were retained as independent predictors of epicardial adipokines in multiple linear regression analysis (backwards model), with age, sex, BMI, CAD, statin use, and aspirin use as initial independent variables.

System; Applied Biosystems) with commercial primers (CD68 and CD11b; Qiagen) or designed by Primer Express 2.0 (adipoR1/2, VCAM-1, ICAM-1, and E-selectin sequences are included in the supplemental Material). Data are expressed as fold or percentage change compared with the reference category and were analyzed with the $2^{-ΔΔCt}$ method.

Statistical Analyses

Data were analyzed using SPSS, version 15.0 (SPSS UK Ltd), and presented as mean±SD or median (interquartile range) or percentage (text) and as box plots or mean±SEM. Between-depot comparisons were performed with the paired $t$ test or the Wilcoxon test, and between-group comparisons were compared with 1-way ANOVA and post hoc Bonferroni multiple comparisons, after logarithmic transformation if required. For categorical variables, a $\chi^2$ test was used. Correlations were assessed with the Pearson or Spearman correlation coefficient, as appropriate. Multiple linear regression (backwards model) was used to investigate independent predictors of epicardial adipokines. Significance was defined as $P≤0.05$.

Results

Patient Characteristics

The demographic and clinical characteristics of the study participants are shown in Table 1. As expected in a population undergoing cardiac surgery, the prevalence of other comorbidities and cardiovascular risk factors was high. For clinical reasons, use of aspirin and statins was significantly more common in patients with CAD. Circulating metabolic and inflammatory markers are included in the supplemental Table.

Circulating Adipokines

Circulating adipokine levels are shown in supplemental Table I. The leptin concentration was higher in patients with a BMI of greater than 27, both with and without CAD. The adiponectin concentration was lower in the CAD/BMI greater than 27 subgroup compared with the control/BMI of 27 or less subgroup; each of the adiponectin isoforms followed similar total adiponectin trends (supplemental Figure I). Significant and previously reported correlations were noted between adipokines and indexes of obesity, insulin sensitivity, inflammation, and lipid levels (supplemental Material).

Release of Adipokines by EAT

Thirteen cytokines were detected by cytokine protein array in EAT-conditioned media from 19 patients, representative of the 4 groups (ie, IL-6, IL-8, MCP-1, and PAI-1), monocyte migration inhibitory factor (MIF), GROα, IL-1 receptor antagonist, sICAM-1, IL-16, IL-13, RANTES, complement 5α, and granulocyte colony-stimulating factor (>10% of the positive control) (Figure 1). In addition, IL-17E, IL-23, IL-27, chemokine C-C ligand 1, chemokine C-X-C ligands 10 and 11, and soluble triggering receptor expressed on myeloid cells 1 were detected only in some patients (n=1 to 3). Six molecules (IL-6, IL-8, MCP-1, PAI-1, MIF, and GROα) were abundant in EAT-conditioned media from all patients (>50% of positive control) (Figure 1). The same 13 cytokines were detected in SAT cultures (n=3, data not shown).

Of the cytokines previously detected, release of IL-6, MCP-1, PAI-1, RANTES, GROα, and sICAM-1, together with sIL-6R, leptin, and adiponectin, was confirmed by ELISAs. Compared with SAT, EAT secreted significantly higher concentrations of all adipokines, apart from RANTES (supplemental Figure II). Significantly higher concentrations of DNA per gram of EAT suggested more cells in this depot compared with SAT (supplemental Figure III). This finding may account, at least in part, for the greater adipokine release. sICAM-1 levels were close to the limit of detection of the ELISA and, therefore, not further reported.

EAT-Derived Adipokines in Obesity and CAD

The protein array data suggested that epicardial release of RANTES, sICAM-1, IL-13, IL-16, and IL-1 receptor antagonist is increased in patients with CAD and/or a BMI of greater than 27 (Figure 1); however, this method is at best semiquantitative. Therefore, we investigated adipokine release in more patients with ELISAs.

The release of adiponectin by EAT was suppressed both in obesity and CAD, with the highest concentrations seen in patients with a BMI of greater than 27 (Figure 2A). CAD was also associated with increased RANTES release from SAT, independently of BMI (Figure 2B). Subcutaneous release of adiponectin and RANTES showed similar trends. Furthermore, epicardial release of adiponectin and RANTES correlated significantly with systemic levels ($r=0.26 [P=0.05]$ and $r=0.72 [P<0.0001]$, respectively).

Epicardial leptin release was not associated with obesity (or CAD), in contrast to subcutaneous release, which was higher in patients with a BMI of greater than 27. Epicardial IL-6, MCP-1, PAI-1, sIL-6R, and GROα levels also did not differ between the groups.

The more prevalent use of aspirin and statins in patients with CAD and the higher number of females among control patients were accounted for in multiple regression analysis (Table 2). Epicardial adiponectin is independently associated with both obesity and CAD. Furthermore, CAD acts as an independent predictor of RANTES and IL-6 release, whereas statins appear to suppress the release of proinflammatory cytokines (ie, RANTES, IL-6, and MCP-1). Aspirin therapy,
Epicardial Adipokines Induce Adhesion of THP-1 Cells to HCAECs

Having established secretion of both proinflammatory and anti-inflammatory mediators from EAT, their effects on cellular populations implicated in atherosclerosis were then investigated. The treatment of HCAECs with EAT-conditioned media from patients with CAD and a BMI of greater than 27 increased the adhesion of THP-1 cells by 15.1% compared with baseline (P=0.002) (Figure 3A) and the expression of ICAM-1 in HCAECs by 2.8-fold. No changes were seen in VCAM-1 and E-selectin expression (Figure 3B). EAT-conditioned media from the CAD/BMI of 27 or less group did not promote adhesion significantly and did not increase expression of ICAM-1 (Figure 3A and B). EAT-conditioned media from control patients also did not induce adhesion (Figure 3A); the expression of adhesion molecules was not assessed as the result of a shortage of media.

HCAECs express AdipoR1 and AdipoR2 mRNA (at 40% and 19%, respectively, of the hepatic Huh7 cells, major adiponectin targets). AdipoR1 protein was also present in HCAECs (Figure 3C). Supplementation of EAT-conditioned media from CAD/BMI greater than 27 patients, with recombiant globular adiponectin, 1500 ng/mL (levels achievable in adiponectin targets). AdipoR1 protein was also present in HCAECs (Figure 3C). Supplementation of EAT-conditioned media from CAD/BMI of 27 or less group did not promote adhesion significantly and did not increase expression of ICAM-1 (Figure 3A and B). EAT-conditioned media from control patients also did not induce adhesion (Figure 3A); the expression of adhesion molecules was not assessed as the result of a shortage of media.

Epicardial Adipokines Induce Migration of THP-1 Cells, An Effect Exacerbated in CAD

In atherosclerosis, the adhesion of monocytes is followed by migration across the endothelial layer. EAT-conditioned media from the 4 patient groups induced the migration of THP-1 cells across a polycarbonate membrane. Media from the control groups induced migration by 63.4% (BMI ≤27) and 83.3% (BMI >27) of that induced by MCP-1, 20 ng/mL; media from the CAD groups induced migration by 112% (BMI ≤27) and 117% (BMI >27) of that induced by MCP-1, 20 ng/mL (Figure 4). EAT-conditioned media from patients with CAD induced significantly more migration compared with controls (114.8±89.8% versus 74.0±59.4%; P=0.008). Migration was not associated with MCP-1 levels in EAT-conditioned media, but correlated inversely with adiponectin levels (r=−0.25, P=0.02).

Discussion

We investigated the release of adipokines by EAT and their potential to interact with cells implicated in atherosclerosis in which is discontinued 1 week before surgery, was not associated with cytokine release.

Given that infiltrating macrophages account significantly for cytokine secretions from adipose tissue, we evaluated mRNA expression of 2 macrophage markers (CD68 and CD11b) in whole EAT samples. No significant differences were noted among the 4 study groups. However, CD68 levels correlated positively with MCP-1 secretion from EAT (r=0.48, P=0.03), and CD11b levels correlated negatively with adiponectin secretion (r=−0.56, P=0.005).

In summary, obesity and CAD may differentially regulate the balance between proinflammatory and anti-inflammatory EAT products. Obesity is primarily associated with changes in adiponectin, whereas CAD is associated with changes in both adiponectin and RANTES.
Epicardial Adipokines Interact With HCAECs

Characterization of the secretions from EAT enables the investigation of their putative role(s) in atherosclerosis. In animal models, periadventitial production of adiponectin and proinflammatory cytokines affected endothelial function in vivo. Given that analogous studies are not feasible in humans, we evaluated the effects of EAT-conditioned media on HCAECs in vitro. Coronary artery cells, as opposed to other endothelial cells, were used for these experiments because their specific anatomical origin modulates endothelial responses. Treatment with EAT-conditioned media from patients with CAD and a BMI of greater than 27 induced expression of ICAM-1 in HCAECs, leading to enhanced adhesion of mononuclear cells to the surface of these cells. Supplementation of the conditioned media with recombinant adiponectin inhibited monocyte adhesion, suggesting that diminished secretion of this adipokine may account, at least in part, for these findings. Indeed, adiponectin has previously been shown to inhibit cytokine-induced expression of adhesion molecules and monocyte adhesion, via inhibition of nuclear factor–κB signaling.

Furthermore, we showed that EAT-conditioned media have chemotactic effects and induce migration of mononuclear THP-1 cells, effects that are exacerbated in the presence of CAD. MCP-1, the prototype monocyte chemoattractant, and other chemokines also implicated in monocyte trafficking (GROα, MIF, and RANTES) are abundant in EAT-conditioned media and may, in concert, mediate this effect. However, the magnitude of migratory response was associated with the levels of adiponectin, rather than the proinflammatory load. Thus, the increased migration noted in those with CAD may owe more to suppressed anti-inflammatory signals. Overall, adiponectin appears to be an important modulator of the effects of EAT in the initiation of atherosclerotic processes and may represent an important future therapeutic target.

Limitations of the Study

Anatomical and ethical restrictions make EAT accessible only in patients undergoing cardiac surgery. This limits our ability to perform longitudinal or comparative studies of EAT with other abdominal visceral adipose tissue depots from the same patient. CAD is an absolute indication for aspirin and statin treatment. These agents have anti-inflammatory effects on adipose tissue and were less frequently used by control patients; their effects could only be accounted for statistically. Finally, this study focused on a white nondiabetic cohort to avoid the confounding effects of ethnicity and diabetes and its accompanying pharmacotherapies on adipose tissue; therefore, the results need to be further ascertained in other ethnic groups and in patients with type 2 diabetes.

In conclusion, we identified several novel inflammatory mediators (eg, GROα, IL-8, MIF, and sICAM-1) as part of the EAT secretome. In a well-characterized human cohort, which included patients without CAD and across the adiposity spectrum, it is apparent that the secretory profile of EAT is altered in those with both obesity and CAD. More important, this study highlights the direct pathophysiologic significance of EAT-generated adipokines in atherosclerosis. In obesity and CAD, epicardial adipokines can induce cell surface expression of adhesion molecules, enhance adhesion of monocytes to endo-

CAD. In addition, IL-6, sIL-6R, and MCP-1 were also secreted by this tissue, as previously described in individuals undergoing coronary artery bypass graft surgery. Many of these molecules participate in several stages of the atherosclerotic process: chemotaxis, foam cell formation, smooth muscle cell proliferation and migration, and plaque destabilization. Therefore, it is plausible that EAT, as a source of these mediators, directly surrounding the coronary arteries, can play a role in cardiovascular disease.

Obesity per se was associated with low adiponectin release from EAT. The same was noted for CAD, in agreement with previous reports. Also, CAD, independently of obesity, was associated with increased RANTES release by EAT, a chemokine that contributes to atherosclerotic plaque formation.

Inflammatory pathways, mediated by nuclear factor–κB and Jun N-terminal kinase, are upregulated in EAT in the presence of CAD; whether the same occurs in obesity and whether these mediators regulate adipokine release remain to be investigated.

Our data suggest that statins suppress cytokine release by EAT. A previous study, which found no associations between medication and proinflammatory EAT secretions, included only patients with CAD. Despite medication, obesity and CAD alter adiponectin, RANTES, and IL-6 levels and contribute to a proinflammatory environment in the vicinity of the coronary arteries.

Figure 4. Migration of THP-1 cells in response to EAT-conditioned media. A, Migration of THP-1 cells in response to serum-free media; MCP-1, 20 ng/mL; and EAT-conditioned media from control/BMI of 27 or less, control/BMI of greater than 27, CAD/BMI of 27 or less, and CAD/BMI of greater than 27 groups, as percentage of MCP-1 (n = 2 to 6 in 6 separate experiments). B, Representative photographs of cells migrating in response to serum-free media (i); MCP-1, 20 ng/mL (ii); EAT-conditioned media from the control/BMI of 27 or less group (iii); and the CAD/BMI of greater than 27 group (iv).

Our data suggest that statins suppress cytokine release by EAT. A previous study, which found no associations between medication and proinflammatory EAT secretions, included only patients with CAD. Despite medication, obesity and CAD alter adiponectin, RANTES, and IL-6 levels and contribute to a proinflammatory environment in the vicinity of the coronary arteries.
thelial cells, and facilitate migration of adherent monocytes. Animal studies suggest that EAT products might also modulate atherosclerosis via regulation of endothelial nitric oxide synthesis and vascular smooth muscle cell function. Other emerging data demonstrate effects of adipokines on cardiomyocytes. Thus, the interactions between EAT-derived adipokines and neighboring cells and organs may represent future therapeutic targets for cardiovascular disease.

Sources of Funding
This study was supported in part by Heart Research UK (RG 25809/09/11) and the European Commission (LSHM-CT-2004-005272).

Disclosures
None.

References
Epicardial Adipokines in Obesity and Coronary Artery Disease Induce Atherogenic Changes in Monocytes and Endothelial Cells
Kalypso Karastergiou, Ian Evans, Nicola Ogston, Nazar Miheisi, Devaki Nair, Juan-Carlos Kaski, Marjan Jahangiri and Vidya Mohamed-Ali

Arterioscler Thromb Vasc Biol. published online April 15, 2010;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 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/early/2010/04/15/ATVBAHA.110.204719.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2010/04/15/ATVBAHA.110.204719.DC1

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/
Supplement Material

Methods

Blood and Adipose Tissue Sampling

Fasting glucose, total cholesterol, triglycerides and HDL-cholesterol were analysed by standard techniques. Lipoprotein a, apolipoprotein A-I and B were assessed by immunonephelometry (in a BN ProSpec Nephelometer, Siemens Healthcare Diagnostics, Surrey, UK) and C-reactive protein (CRP) levels with an immunoturbidometric method (in a Beckman Synchron LX-20 pro autoanalyzer, Beckman Coulter, Buckinghamshire, UK). All assays had inter- and intra-assay coefficients of variation of <10%. LDL-cholesterol concentration was calculated with the Friedewald formula. Insulin levels were assessed by ELISA (Mercodia, Sweden) and insulin resistance by the Homeostasis Model Assessment Index–IR (HOMA-IR).

Assessment of adiponectin isoforms

Adiponectin isoforms were studied in serum samples representative of the four groups (n=7-15 per group) with a native gel electrophoretic assay, as described before with modifications. Briefly, diluted samples (1:50 in distilled water and 1:2 in sample buffer) were loaded on to a native gel (NativePAGE 4-16% Bis-Tris Gel, Invitrogen, Paisley, UK). Electrophoresis was conducted at room temperature in Native PAGE running buffer (Invitrogen) at 150 V for 3.5h followed by 200V for 2.5h. Subsequently, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen) overnight (4°C, 15V). Proteins were then fixed with incubation in acetic acid (8% v/v for 15 min) and membranes saturated in 5% non-fat
milk in PBS/1% Tween 20 at room temperature for 1h. Following incubation with a mouse monoclonal antibody against human adiponectin (BD Biosciences, Oxford UK, at 1:10000 in 1% milk in PBS/T for 1h at room temperature) and with a horseradish peroxidase-linked sheep antibody to mouse IgG (GE Healthcare, Little Chalfont, UK, at 1:3000 in similar conditions), membranes were incubated with enhanced chemiluminescence reagents (GE Healthcare, UK), exposed to x-ray film (Amersham Hyperfilm ECL, GE Healthcare, UK) for 5-30 seconds and the film was developed. Four bands were identified with this approach: high-molecular weight (HMW) at 750 kDa, middle-molecular weight (MMW) at 270 kDa and two low-molecular weight forms (LMW1 and LMW2) at 130 and 60 kDa respectively. Serum sample of the same healthy volunteer was used as control in all measurements. Bands were quantified with Image J. For every patient, each band was expressed as % of the same band in the control (healthy volunteer) sample. Due to shortage of material, these measurements were not performed in the EAT-conditioned media.

**Human Coronary Artery Endothelial Cell Culture**

HCAECs (TCS Cellworks, Park Leys, Bucks, UK) were cultured in endothelial basal medium with 10 ng/mL human epidermal growth factor, 12 μg/mL bovine brain extract, 50 μg/mL gentamycin sulphate (Cambrex Bio Science, Wokingham, UK) and 10% (v/v) foetal bovine serum (FBS, Autogen Bioclear, Wilts, UK). HCAECs, between passages 2-4 and at 80–90% confluency, were maintained at 1% serum-containing medium for 12h, followed by 12h incubation in EAT-conditioned media. Cells were used for RNA extraction and cDNA synthesis and vascular cell adhesion molecule-1 (VCAM-1), ICAM-1, E-selectin mRNA expression was assessed by real time RT-PCR. Unstimulated HCAECs were used to assess mRNA and expression of
adipoR1 and adipoR2, with Huh7 cells (ATCC, Middlesex, UK), a human hepatoma cell line, as positive controls.

Primer sequences were as follows: AdipoR1 forward 5´-
GCCAGATGGGCTGGTTCTT-3´ and reverse 5´-CATAAAGGCCAGCTCCAGTGA-3´; AdipoR2 forward 5´-TGTTCACTTCCATGGTGTC-CAA-3´ and reverse 5´-GCCCCCGCCGATCAT-3´; VCAM-1 forward 5´-AAGATGGCTGATCCTTGG-3´ and reverse 5´-GGTGCTGCAAGTCAATGAGA-3´; ICAM-1 forward 5´-CCGAGCTCAAAGTCTAAAG-3´ and reverse 5´-TGCCACCAATATGGGAAGGC-3´, E-selectin forward 5´-AGCTTCCAAGTCAATGAGA-3´ and reverse 5´-GGCTCAGGCAGCTCGTAGCTC-3´.

Protein expression of AdipoR1 was assessed in HCAECs with sodium dodecyl sulphate polyacrylamide gel electrophoresis, followed by blotting onto polyvinylidene fluoride membranes and probing with polyclonal antibodies against human AdipoR1 (SantaCruz Biotechnologies, Wembley, UK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ABD Serotec, Kidlington, UK). Antigen–antibody complexes were detected by chemiluminescence (Amersham Biosciences, Little Chalfont, UK).

**Adhesion Assay**

HCAECs (between passages 2-4) were grown in 96-well plates; at confluency and after 12h in 1% serum-containing medium, they were treated with EAT-conditioned medium (6h). THP-1 cells, a human monocytic cell line (ATCC, Middlesex, UK), were cultured in RPMI 1640 with 10% FBS and 0.05 mM 2-mercaptoethanol and used
between passages 3-5. They were labelled with fluorescein (5 μM; BCECF-AM, Invitrogen, Oregon, USA) for 30 minutes, added to HCAECs (10⁶/mL) and allowed to adhere for 60 minutes. Non-adherent cells were washed out and fluorescence measured with a fluorescence plate reader (GENios, TECAN, UK) after cell lysis with Triton-X (1%). TNFα (50 ng/mL) was used as positive control and it increased adhesion by 24%.

**Migration assay**

The bottom wells of a 48-well Boyden chamber (Receptor Technologies, Warwickshire, UK) were filled with 26 μL of serum-free media, EAT-conditioned media, or 20 ng/mL MCP-1 (as positive control, Invitrogen, UK). A polycarbonate membrane with 5 μm pores and a silicon gasket were placed on top. The top wells were filled with 52 μL of THP-1 cells (10⁶/mL). The apparatus was incubated for 1h (37°C/ 5% CO₂). After removing non-migrated cells by drawing the top filter side over the edge of a windshield wiper, the filter was fixed by immersion in methanol and dried. Migrated cells were stained with a Quick-Diff kit (Reagena, Toivala, Finland). Filters were observed under an AxioImager A1 microscope (Carl Zeiss, Herts, UK) and images captured by an AxioCam MRc5 camera (Zeiss). Cells were counted in three fields per well with a 10X objective.
Results

Circulating Adipokines

Leptin concentration was higher in patients with BMI>27 kg·m\(^{-2}\), both with and without CAD (Supplementary Figure IA). Adiponectin concentration was lower in the CAD/BMI>27 kg·m\(^{-2}\) subgroup in comparison to the control/BMI≤27 kg·m\(^{-2}\) one (Supplementary Figure IB). All adiponectin isoforms mirrored differences of total adiponectin with the highest concentrations found in the control/BMI≤27 kg·m\(^{-2}\) group (Supplementary Figure IC and data not shown). No significant differences were noted between the four subgroups in systemic concentrations of the other adipokines.

Leptin levels correlated with BMI (r=0.64, \(P<0.001\)), waist circumference (r=0.35, \(P=0.05\)) and HOMA-IR (r=0.65, \(P=0.05\)). Adiponectin levels correlated negatively with waist circumference (r=-0.48, \(P=0.04\)) and positively with HDL-cholesterol (r=0.37, \(P=0.02\)) and apolipoprotein A-I (r=0.54, \(P=0.001\)). Furthermore, PAI-1 levels correlated with BMI (r=0.45, \(P=0.05\)), IL-6 with CRP (r=0.63, \(P<0.001\)) and RANTES with insulin resistance (HOMA-IR, r = 0.38, \(P=0.05\)). Female sex was associated with higher levels of adiponectin [11.1 (8.6-19.8) versus 9.3 (3.7-12.9) µg/mL, \(P=0.03\)], leptin [19.4 (13.2-46.8) versus 8.5 (4.4-14.3) ng/mL, \(P<0.001\)] and IL-6 [4.8 (2.3-8.3) versus 2.3 (1.4-6.0) pg/mL, \(P=0.02\)]. No associations were noted between circulating adipokines and blood pressure or history of hypertension, smoking and family history of CAD.
References


Figure Legends

**Figure I.** A. Circulating concentrations of leptin and B. adiponectin in control/BMI≤27 kg·m⁻², control/BMI>27 kg·m⁻², CAD/BMI≤27 kg·m⁻² and CAD/BMI>27 kg·m⁻² patients. C. HMW-adiponectin levels in control/BMI≤27 kg·m⁻² (n=7), control/BMI>27 kg·m⁻² (n=10), CAD/BMI≤27 kg·m⁻² (n=7) and CAD/BMI>27 kg·m⁻² (n=15) patients, as % of HMW-adiponectin of the same healthy volunteer. Boxplots represent median, IQR, range and outliers.

**Figure II.** Paired EAT and SAT release of leptin (A), adiponectin (B), IL-6 (C), sIL-6R (D), RANTES (E), MCP-1 (F), PAI-1 (G) and GROα (H) (ng or pg per gram of tissue per hour of culture). Boxplots represent median, IQR, range and outliers.

**Figure III.** DNA content of SAT and EAT used in organ cultures from n=18 patients, representative of the four study groups. Boxplots represent median, IQR, range and outliers.
### Supplementary Table I. Circulating levels of metabolic/inflammatory markers and adipokines

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control group</th>
<th>CAD group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMI≤27 kg·m⁻²</td>
<td>BMI&gt;27 kg·m⁻²</td>
<td>BMI≤27 kg·m⁻²</td>
</tr>
<tr>
<td>N</td>
<td>20</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>169±54</td>
<td>188±48</td>
<td>133±32</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>69 (52–92)</td>
<td>59 (41–76)</td>
<td>44 (36–58)</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>131 (81–155)</td>
<td>131 (120–184)</td>
<td>101 (70–124)</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>91 (75–140)</td>
<td>223 (151–265)</td>
<td>115 (72–193)</td>
</tr>
<tr>
<td>Lp(a) (g/L)</td>
<td>0.06 (0.04–0.15)</td>
<td>0.14 (0.03–0.42)</td>
<td>0.09 (0.02–0.56)</td>
</tr>
<tr>
<td>apoB (g/L)</td>
<td>0.67±0.18</td>
<td>0.78±0.28</td>
<td>0.70±0.27</td>
</tr>
<tr>
<td>apoA-I (g/L)</td>
<td>1.40±0.29</td>
<td>1.46±0.33</td>
<td>1.29±0.27</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>84±12</td>
<td>97±25</td>
<td>88±11</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>26.5±13.3</td>
<td>56.7±42.8</td>
<td>38.7±23.5</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.81±0.42</td>
<td>2.13±1.84</td>
<td>1.24±0.78</td>
</tr>
<tr>
<td>hs-CRP (mg/dL)</td>
<td>3.7 (3.4–10.9)</td>
<td>4.2 (0.7–8.8)</td>
<td>3.5 (1.6–3.8)</td>
</tr>
<tr>
<td>Serum leptin (ng/mL)</td>
<td>7.2 (3.1–15.6)</td>
<td>13.6 (9.0–50.3)</td>
<td>6.7 (3.2–15.7)</td>
</tr>
<tr>
<td>Serum adiponectin (μg/mL)</td>
<td>12.7 (9.1–20.3)</td>
<td>10.2 (9.3–12.8)</td>
<td>12.7 (7.4–20.8)</td>
</tr>
<tr>
<td></td>
<td>Control/BMI ≤ 27 kg·m(^{-2})</td>
<td>Control/BMI &gt; 27 kg·m(^{-2})</td>
<td>CAD/BMI ≤ 27 kg·m(^{-2})</td>
</tr>
<tr>
<td>-------------------------</td>
<td>--------------------------------</td>
<td>---------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Serum IL-6 (pg/mL)</td>
<td>4.2 (1.4–17.1)</td>
<td>4.8 (1.7–6.1)</td>
<td>2.5 (1.3–4.7)</td>
</tr>
<tr>
<td>Serum sIL-6R* (ng/mL)</td>
<td>45.1±14.5</td>
<td>53.1±6.9</td>
<td>50.7±13.5</td>
</tr>
<tr>
<td>Serum MCP-1* (pg/mL)</td>
<td>165 (106–353)</td>
<td>226 (186–367)</td>
<td>234 (155–322)</td>
</tr>
<tr>
<td>Serum RANTES* (ng/mL)</td>
<td>23.5±25.4</td>
<td>44.0±27.1</td>
<td>60.7±35.1</td>
</tr>
<tr>
<td>Serum PAI-1* (ng/mL)</td>
<td>3.3 (2.4–5.2)</td>
<td>5.6 (4.3–6.6)</td>
<td>3.5 (2.3–7.2)</td>
</tr>
</tbody>
</table>

HOMA-IR: Homeostasis Model Assessment – Insulin Resistance, hs-CRP: high-sensitivity C-Reactive Protein, IL-6: interleukin-6, sIL-6R: soluble IL-6 Receptor, MCP-1: Monocyte Chemoattractant Protein-1, RANTES: Regulated Upon Activation Normal T-cell and Secreted.

Data presented as mean±SD or median (IQR). P values represent comparisons between control/BMI≤27 kg·m\(^{-2}\), control/ BMI>27 kg·m\(^{-2}\), CAD/BMI≤27 kg·m\(^{-2}\), CAD/BMI>27 kg·m\(^{-2}\) subgroups with one-way ANOVA or Kruskal-Wallis. * n=5-10 per group.
Figure 1.
Figure II.

A. Leptin (ng/g/h)

B. Adiponectin (ng/g/h)

C. IL-6 (ng/g/h)

D. sIL-6R (pg/g/h)

E. RANTES (pg/g/h)

F. MCP-1 (ng/g/h)

G. PAI-1 (ng/g/h)

H. GROα (ng/g/h)

** p≤0.01
*** p≤0.001
x: outlier
Figure III.